

AN ELECTROPHYSIOLOGICAL STUDY OF PUTATIVE  
NEUROTRANSMITTERS AND HISTOCHEMICAL  
LOCALISATION OF BIOGENIC AMINES IN THE  
CENTRAL NERVOUS SYSTEM OF THE COCKROACH  
'PERIPLANETA AMERICANA'

Jean Roy Fleming

A Thesis Submitted for the Degree of PhD  
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AND HISTOCHEMICAL LOCALIZATION OF BIOGENIC AMINES IN THE  
CENTRAL NERVOUS SYSTEM OF THE COCKROACH,  
PERIPLANETA AMERICANA

A thesis submitted to the University  
of St. Andrews for the degree of  
Doctor of Philosophy

by

JEAN ROY FLEMING



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## ABSTRACT

A modification of the fluorescent histochemical glyoxylic acid (GA) method was used for localization of biogenic amines in wholemount ventral nerve cord and suboesophageal ganglia of the cockroach, Periplaneta americana. The distributions of fluorescent neurone somata within each ganglion and of nerve processes within the ganglia, interganglionic connectives and peripheral nerves were determined. An assessment was made of the effects of colchicine, nialamide plus DOPA and reserpine on the specific tissue fluorescence. The available evidence suggests that most of the specific fluorescence is due to dopamine.

Infusion of hexaminecobaltic chloride was used to identify which fluorescent neurone pairs in the prothoracic and suboesophageal ganglia projected into the interganglionic connectives and peripheral nerves. In order to determine the detailed morphology of certain of the fluorescent neurones GA was combined with intracellular injection of either Lucifer Yellow, hexaminecobaltic chloride, or Horseradish peroxidase. Technical difficulties, however, were encountered with the identification and filling of the neurones.

A prothoracic neurone was identified which depolarized in response to pressure ejected and bath applied dopamine and noradrenaline. Both phentolamine and propranolol reversibly inhibited the dopamine response, although these drugs were also effective at antagonizing the cell's response to

acetylcholine. Bath application of the drugs forskolin, IBMX and dibutyryl cyclic AMP provided evidence that in this case dopamine does not act through the second messenger, cyclic AMP.

Additional electrophysiological experiments determined the effect of pressure ejected application of glutamate and GABA upon the electrical response of an identified metathoracic motoneurone. Picrotoxin reversibly inhibited the amino acid responses. Long term application of both picrotoxin and 4-acetamido-4'-isothiocyano stilbene-2,2'-disulphonic acid (SITS) produced changes in the membrane properties of the neurone such that the neurone somata became able to actively support action potentials.



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GENERAL INTRODUCTION

GENERAL INTRODUCTION

This chapter is split into several sections, each dealing with a different aspect of insect biology. The first covers the origin of insects and emphasizes their supremacy as world colonizers over other animal groups. This is followed by a short account of the enormous importance of insects to man and his dilemma in dealing with them since they cause much growth but also much destruction in the environment. The second section gives a brief summary of the organization of the insect nervous system. This provides good background information for the experimental sections and shows the advantages of a simple nervous system in the study of neurobiology.

Following on from this there are four short sections dealing with areas of insect neurobiology, namely the development of the nervous system and the neural basis of behaviour, which are providing answers of relevance to the much more complicated field of mammalian neurobiology. Work on insects, and invertebrates in general, is often maligned since it is not seen to be of value in the understanding of the working of the mammalian (especially human) body. With neurobiology, however, much of the important ground work has been laid using simple invertebrate preparations (the prime example being the squid axon). In addition, the work in this thesis is concerned with the distribution of catecholamine-containing neurones in the cockroach ventral nerve cord. The factors that determine which neurotransmitter is used by a particular neurone are not yet known, although work on the development of the grasshopper nervous system is starting to

provide some clues. With regard to the neural basis of behaviour, the electrophysiology and functional connections of certain groups of neurones are known, but nothing is understood about the pharmacology of the synapses between them. To obtain a complete picture of a functioning nervous system it is necessary to study all three areas described above, with the ultimate aim being to know the origin of a specific neurone, the neurotransmitter it uses, the functional connections it makes, and the behaviour in which it has a crucial role. Hence it was considered worthwhile to mention some of the recent important results in the fields of insect nervous system development and the neural basis of behaviour to complement the work in this thesis on the electrophysiology and pharmacology of insect neurones and to give a more complete picture of what is known about the development and working of the insect nervous system as a whole.

The final three sections are concerned with the work which is directly related to this thesis and covers the pharmacology and electrophysiology of insect nervous system synapses. The main groups of putative transmitters - biogenic amines, acetylcholine and amino acids - are each covered in a separate section which summarizes what is known about the distribution of the drug and its receptors and their role in the functioning of the nervous system.



INSECTS - THEIR ORIGINS AND THEIR IMPACT ON HUMAN AFFAIRS

The Class Insecta is known with certainty in fossil form only from about 300 million years ago. At this stage most of the orders represented were already winged and showing great diversity. Since the advent of winged insects must have been preceded by a period when only wingless ones occurred, their true origin must lie much further back, perhaps around 400 million years ago. This is an extremely long period, during which groups of comparable antiquity have failed to diversify to such an extent, whereas others have simply become extinct.

It is fairly certain that insects originated and evolved as terrestrial animals, but it is very definite that they have achieved a remarkably successful conquest of all non-marine parts of the globe. It has been estimated that there may be as many as three million species of insect, with only a third of these having been described so far. Hence, there could be up to ten times as many insect species as there are in the whole of the the rest of the Animal Kingdom put together. They have been found to inhabit hot springs and ice-cold water; equatorial rain forest and desert; high alpine regions and sea-level areas; even Himalayan glaciers, where a new species of midge has just been discovered. This success of insects can be attributed to a number of factors: (i) The development of a tracheal system; and (ii) the acquisition of a virtually impermeable cuticle. These two developments cut out water loss to a high degree and so enable insects to inhabit very dry regions. Water loss (through sweating and evaporation from the lungs) is the

crucial limiting factor for the colonization by birds and mammals. Reptiles, the other great desert dwellers, can only thrive in climates with a reasonably warm ambient temperature and so their numbers rapidly decline towards the most southern and northern parts of the world.

(iii) The development of wings. Insects are only rivalled by birds in the ability of one group to spread throughout the globe.

(iv) The development of the process of metamorphosis. This has meant that many species are able to inhabit a much wider range of habitats than if the adult and larva were identical.

Out of all the different land based groups in the animal kingdom it is only insects that are able to inhabit the whole range of land climates and this has led to their superiority of numbers.

Insects are of great ecological significance with two thirds of all flowering plants being dependent on insects for pollination. The principal pollinators are bees, wasps, butterflies, moths, and flies, and the evolutionary history of these is in fact closely tied to that of the flowering plants.

Insects are of enormous importance to man, both as friend and foe. Some of the flowering plants which depend on pollination are very important food crops. Although many of these plants will self-pollinate, insect pollination gives increased yield, an earlier and more uniform crop, and also an increase in the quality of the fruit or seeds. On the

other hand, many of our cultivated plants are destroyed by insects. There are species that attack fruits, leaves, stems and roots. A plague of locusts can devour a farm's crop in a few hours. All arid regions of the world are inhabited by locusts and grasshoppers, and since some of these regions have subsistence farming, famine and real hardship can result from a plague. For the more affluent regions locusts cause a substantial financial drain through the many millions of pounds that are lost each year in crop losses and control measures. But it is not just growing food plants that are attacked. Forests are defoliated; timber buildings are weakened and destroyed; household goods are attacked; stored cereals, dried fruit, spices and tobacco serve as breeding grounds. It has been said that up to 50% of the maize stored on North American farms may be destroyed by insects in some seasons. Apart from intensive agriculture creating conditions favourable to insect crop pests, the increased travel and commerce which have also resulted has led to the introduction of pests into new countries where, since they no longer have their native enemies, they flourish.

Many species have become ecto- or endoparasites of vertebrates. Fleas, lice and bed-bugs are examples of long-term external parasites; internal ones include the maggots of various flies which are normally found in animals such as cattle, sheep and horses, but which can get into the human body. Fortunately, these parasitic insects do not often carry human disease. Outstanding exceptions are bubonic plague which is carried by rat-fleas and typhus and

relapsing fever which are carried by lice. With regard to man's livestock, the irritation set up by parasitic insects can be very debilitating, and the wounds and sores that they cause may easily become infected with bacteria. Also, some of the fly maggots can be dangerous since they are able to destroy quite a lot of the living tissue of their host. The most important groups of parasitic insects, with respect to man, are the blood-sucking ones, those which only prey on the host for food. Of these, the most feared are the mosquitoes. The diseases they carry include malaria in all its forms; infections by parasitic worms called filariasis, elephantiasis being one result; yellow fever, and a number of other viral diseases. Another notable member of this group is the tsetse fly which transmits sleeping sickness. Even the common housefly can be dangerous and has been known to transmit typhoid fever and dysentery. (See Oldroyd, 1960; Barnes, 1980; Free, 1970; Waterhouse and Norris 1980; Hinton, 1977; Wigglesworth, 1976; Koshima, 1984).

As a result of this impact of insects on human affairs vast sums of money are spent on the control of insect pests, and on the development of insecticides that are lethal to the pests but harmless to the rest of the animal population, including their own insect parasites.

From the above the importance of studying insects in general simply for their own sake is clearly seen. But insects are also useful in the study physiology in general. In 1939 Sir Vincent Wigglesworth said, "Insects provide an ideal medium in which to study all the problems of physiology." This is especially true of the nervous system, whether it be with

regard to nervous system organization or development or integration or to the behaviour that results. The attraction of the insect nervous system for many neurobiologists is that it is relatively simple, at least in terms of numbers of neurones. In the next section the organization of the insect nervous system will be described, along with the advantages it offers. Then in the four following short sections a few of the areas in which study of the insect nervous system has, and is, playing a prominent role are mentioned along with their major discoveries.

THE ORGANIZATION OF THE INSECT NERVOUS SYSTEM

The central nervous system of an insect consists of a brain and a series of ganglia. Early in embryogenesis each body segment has a segmental ganglion, but as development proceeds some fusion of ganglia takes place leading to the final adult form. For example, the suboesophageal ganglion, which innervates the mouth parts consists of three fused embryonic ganglia. All the ganglia are bilaterally symmetrical and are linked by longitudinally paired interganglionic connectives. This means that most cells have an identical contralateral partner and this can be used as a control cell in certain experiments.

Fig. I 1 illustrates the main features of ganglionic organization. The whole central nervous system is surrounded by a nerve sheath. This is made up of an outer acellular neural lamella, consisting of collagen-like fibres in a matrix of mucopolysaccharides, and an inner perineurium, consisting of a layer of specialized glial cells. Beneath this is the glial cell layer, in which lie the cell bodies of the neurones. Most of these lie ventrally, with only a few on the dorsal surface. The suboesophageal ganglion contains about 5000 neurones; the three thoracic ganglia about 3000 neurones each; and the abdominal ganglia about 500 each. The cell bodies of individual neurones occupy characteristic positions and this has allowed the production of neurone maps for certain of the ganglia (see Cohen and Jacklet, 1967; Young, 1969; Iles, 1976). The central core of the ganglion, called the neuropile, is mainly composed of

Figure I.1 Diagram of a transverse section through a typical ganglion in the insect ventral nerve cord. The neurone cell bodies are located around the periphery of the ganglion. The neuropile consists of an extremely complex network of processes from many thousands of neurones, whose cell bodies may be located within that ganglion, in the periphery or within a more anterior or posterior ganglion.

DORSAL

Principal interneurone axon and dendrites

Neuropile

Cell body layer

Sensory axon

Peripheral nerve

Nerve  
sheath



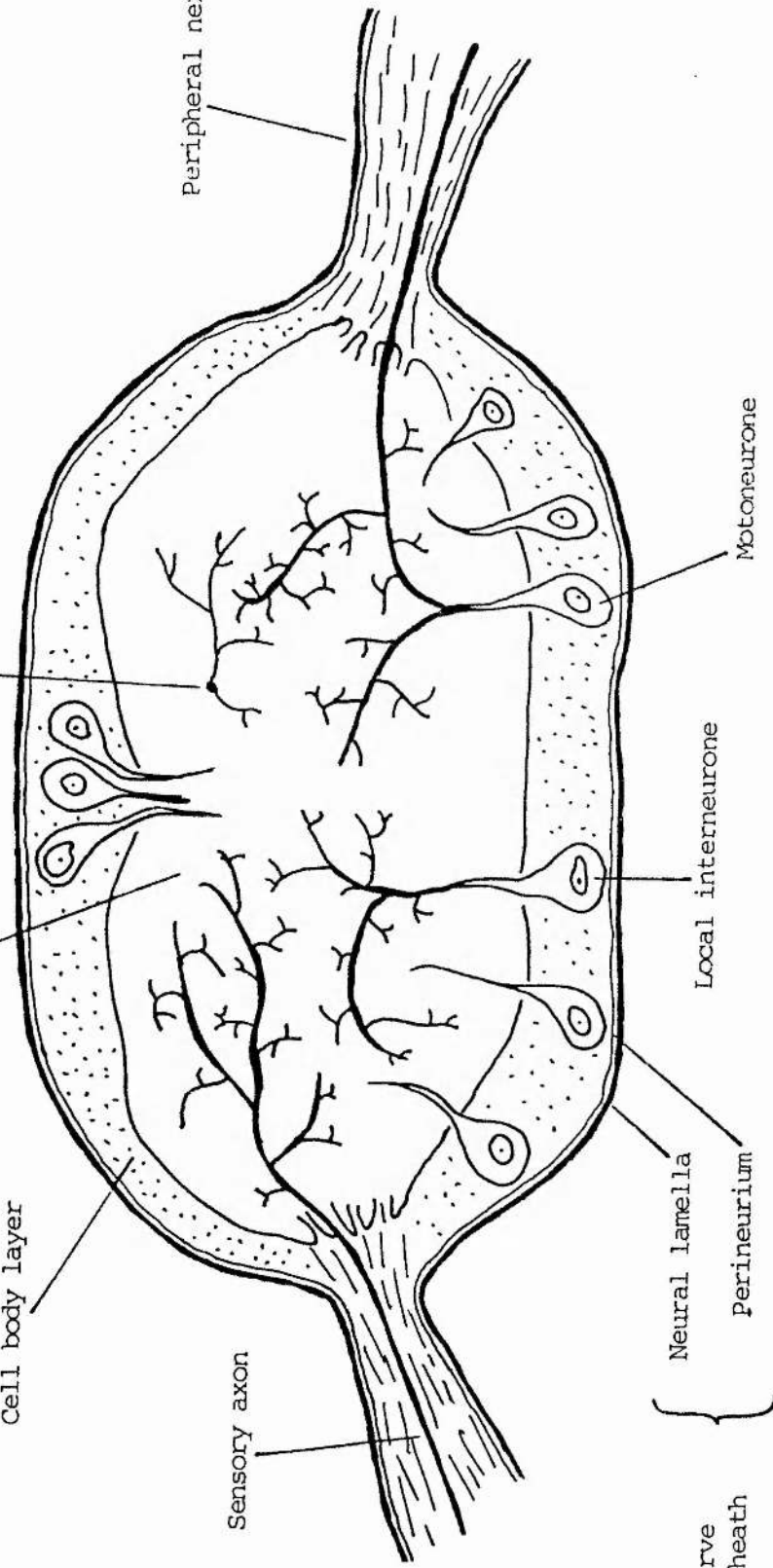
Neural lamella

perineurium

Local interneurone

Motoneurone

VENTRAL





axons and dendrites and the processes of glial cells which surround them. It is in the neuropile that synaptic connections between axonal and dendritic branches of the neurones are made. No synaptic contacts are found on the neurone cell bodies or in the interganglionic connectives, which only contain axons. These axons can extend the whole length of the nerve cord, or simply run between adjacent ganglia. (See Gregory, 1974, for a more comprehensive summary of ganglionic structure; and Lane, 1974, for a review of the tissue components in detail.) The organization of the nerve fibres within the neuropile is in fact much more structured than is illustrated in the simple diagram of Fig. I.1 and ideas concerning this are summarised below.

The neuropile of a ganglion was originally considered to be divided into three layers:

1. Sensory neuropile - located ventrally.
2. Motor neuropile - located dorsally.
3. An interneuronal association area lying between these two. Sensory input feeds into this area and its output drives the motoneurones.

Due to work on the neuroanatomy of sensory and motoneurones Altman (1980) suggested a modification of this as follows:

1. A part of the ventral neuropile is exclusively sensory, containing fibre endings from inputs which need to be analyzed spatially and integrated (for example, input from tactile hairs). This Altman (1980) termed the sensory integration neuropile.
2. There is no separate association neuropile, with information for motor output being processed in areas

containing motor fibres. These she termed sensori-motor integration neuropiles and they make up the bulk of the total neuropile area. In addition, Altman (1980) suggests that each motor output pattern or behaviour is represented by a discrete area within the sensori-motor neuropile and that dual function neurones will branch to each functional area.

Insect neurones can be usefully divided into at least five classes: motoneurones, sensory neurones, local interneurones, principal interneurones and neurosecretory neurones. These classes are not meant to be mutually exclusive and some neurones may be placed in more than one. Their main features are as follows.

(i) Motoneurones. In general these are unipolar, have central cell bodies and are located in the ganglion of the body segment which is innervated. All have complex arborizations within the neuropile. Motoneurones were originally thought to be simply output elements controlling muscle force. It was then shown physiologically, however, that locust flight and leg motoneurones can influence other neurones within the ganglion (Burrows, 1973; Hoyle and Burrows, 1973), although there is little evidence for direct contacts between motoneurones themselves, except in the case of an excitatory input from the fast extensor to the slow extensor motoneurone in the tibiae of the locust (Heitler and Burrows, 1977). More recently, detailed morphological and ultrastructural studies have been carried out on a few locust motoneurones at both the light and electron

microscopic level (Watson and Burrows, 1981 and 1982; Watson, Burrows and Hale, 1985). It has been found that the metathoracic fast extensor tibiae motoneurone (FETi) and the mesothoracic tergosternal flight motoneurones have both input and output synapses on their ganglionic neurites, and that these are involved in serial, reciprocal and recurrent relationships. Other motoneurones, however, (for example, the slow extensor and the fast flexor tibiae motoneurones and the CI1 common inhibitory motoneurone) have only output synapses (Watson and Burrows, 1981 and 1982; Watson Burrows and Hale, 1985).

(ii) Sensory neurones. There are many more sensory neurones than motoneurones. Most, but not all (Braunig and Hustert, 1979), have peripheral cell bodies with processes that extend into the central nervous system. The projections of the sensory neurones into the central nervous system can be very complex (see for example Altman and Tyrer, 1977) and can extend into more than one ganglion (Hustert, 1978). Sensory neurones can synapse directly onto motoneurones (Burrows, 1975) and also have been shown to have post- as well as presynaptic connections (Altman, Shaw and Tyrer, 1979 and 1980).

(iii) Principal interneurones. These interneurones channel information from one part of the nervous system to a more distant part. Their cell bodies can be; (a) in the brain and their axons descending (for example the descending ocellar neurones of the locust, described by Simmons, 1980); (b) in the last abdominal ganglion and their axons ascending (for example the giant interneurones of the cockroach, first

reported by Pumphrey and Rawdon-Smith, 1937); (c) in other ganglia with their axons descending, ascending or both (for example the thoracic ocellar neurones of the locust, described by Rowell and Pearson, 1983). Although these neurones tend to be named according to the sensory modality through which they are first discovered, they may in fact receive other sensory inputs (for example in 1975 O'Shea reported that the descending movement detector of Acridids also responds to sound).

(iv) Local interneurones. These channel information over a small region of the nervous system. Over recent years a great many of these have been identified (various examples are given below) and it now thought that up to 70% of all cell bodies in a segmental ganglion may be local interneurones (Seigler and Burrows, 1979). They can be spiking or non-spiking; they receive various types of sensory information; they exist in the brain and the segmental ganglia, and have a central role in the generation of behaviour, both sudden and rhythmic (Robertson and Pearson, 1984; Pearson, 1983; Wilson and Phillips, 1982; Burrows, 1980; Pearson and Fourtner, 1975). Some recent work has tried to tackle the problem of why there should be these two distinct types of local interneurone (see review by Burrows, 1985). Burrows (1985) considers that the two groups have distinct functions in local integrative processes. The non-spiking interneurones show a graded and sustained release of transmitter and hence are well suited to producing motor output that can be precisely and smoothly varied. The spiking interneurones, however, provide a means for

integrating the huge initial inflow of sensory information (about 10,000 sensory axons from the locust hindleg alone converge on a few hundred spiking local interneurons in each half of the metathoracic ganglion).

(v) Neurosecretory neurones. These can have their cell bodies in the central nervous system or in the periphery. The corpora cardiaca, for example, contains neurosecretory cells but also contains processes from brain neurosecretory cells. Both sets liberate their products into the blood or into connective tissue near the target. (See Finlayson, 1980, and Evans, 1980a, for reviews about neurosecretory cell distribution, function, and biochemistry.) In many insects the ventral nerve cord ganglia contain a group of dorsally unpaired medial (DUM) neurones. These have been most extensively studied in the locust metathoracic ganglion, where it has been found that at least some, if not all, are octopaminergic (Hoyle, 1975; Hoyle and Barker, 1975; Evans and O'Shea 1977 and 1978; Dymond and Evans, 1979). Both centrally and peripherally it appears that the octopamine, which is released from blindly ending neurosecretory terminals, has modulatory actions (Hoyle, Dagan, Moberly and Colquhoun, 1974; Hoyle, Colquhoun and Williams, 1980; Evans and O'Shea, 1977 and 1978; O'Shea and Evans 1979; Evans 1981, 1984a and b; Sombati and Hoyle 1984a and b; see also pps 40, 41, 41a-c, 158 and 159).

As was stated above, some neurones may not fit into just one category and should be considered as multifunctional. For example, in (i) above it was mentioned that certain motoneurons can influence other neurones as well as control

muscle force. It has also been found that both the coxal depressor motoneurone of the cockroach (Ds) and the slow extensor motoneurone (SETi) of the locust release the peptide proctolin along with the excitatory neuromuscular transmitter glutamate. The proctolin has a more long term modulatory action on the muscles (Bishop, O'Shea and Miller, 1981; O'Shea and Adams, 1981; Adams and O'Shea 1983; O'Shea 1985; see also pps 20-20a). Hence these neurones could be considered as modulatory neurones (like the DUM neurones) as well as motoneurones.

All the above types of neurone are also found in vertebrate nervous systems, but most are not anywhere near as accessible as they are in insects, where many of the cell bodies are on the surface of the ganglia and hence are amenable to intracellular electrophysiological techniques. This accessibility of insect neurones has led to a number of them being called 'identified'. This means that the characteristics of the neurone, with regard to its position, morphology and electrophysiology, are known and enable it to be picked out from all the other neurones in every preparation. Experiments can therefore be repeated consistently on the same neurone(s) and so provide more meaningful results. Also, in insects certain behaviours involve a relatively small number of neurones and so this opens up the possibility of describing complete behavioural circuits, which may help in the understanding of the generation of movement in more complex animals.

Recent work in decapod crustaceans and marine mollusks,



however, suggests that there is no such thing as a unique 'behavioural circuit' but rather that a single anatomical network of neurones can be organized into multiple configurations, each producing a particular behavioural function (Getting and Dekin, 1985; Getting, 1985; Marder, 1985 and 1986; Marder and Hooper, 1985). For example, in Tritonia, the neurones involved in the swim system circuit function in at least two configurations to produce: a) reflexive withdrawal; or b) swimming. The network's preferred state is (a), but it will switch to (b) if there is a large enough increase in the amount of sensory input. This initiates a change in the pattern of connectivity within the network and results in a switch to swimming behaviour (Getting, 1985; Getting and Dekin, 1985).

In decapod crustaceans, the stomatogastric ganglion (STG) contains the neurones which together make up the central pattern generator producing timing of the rhythmic movements of the pyloric region of the stomach. The STG receives input from other ganglia via fibres in the stomatogastric nerve. A large number of different putative transmitters are present in these fibres, and each is capable of producing different and specific changes in the phasing and frequency of the pyloric rhythm. Each acts on a particular subclass of the pyloric rhythm neurones to modulate their voltage dependent conductances and strength of their synaptic connections. One anatomical circuit is therefore capable of acting functionally as if it were many different circuits (Marder, 1985 and 1986; Marder and Hooper, 1985).

Hence it is now clear that to completely explain the underlying mechanism for a particular behaviour much more is needed than a knowledge of the motoneurons involved and their synaptic connections. But this does not denigrate the crucial role that neural circuitry analysis plays in answering questions about behaviour and its plasticity. For example, it was only after the neural circuits for locust flight and jumping had been fairly well understood that it became clear that the afferent inputs were an essential component in producing the appropriate behavioural output (see review by Robertson and Pearson, 1985). This type of approach has also been fundamental in discerning a neural explanation for the higher-order behavioural functions of motivation, associated learning and choice in the mollusk Pleurobronchea (see review by Davis, 1985).



DEVELOPMENT OF A NERVOUS SYSTEM

Studies over the last few years on the development of the grasshopper nervous system, are bringing to light strategies that may well be used to generate complex neural networks from the much simpler initial starting structures. The grasshopper has been used because its embryo offers certain special advantages: (a) due to their transparency, individual neurone precursors can be identified and watched divide and giving birth to neurones, hence the precise lineage of a cell can be determined; and (b) the nervous system is in a superficial position and so the neurones (and their precursors) can be impaled with microelectrodes, thus allowing their electrophysiology and morphology to be determined during the various stages of development.

The key points established so far are as follows:

(i) From the neuroepithelium in each segment of the embryo, there arise 68 precursor cells. These are arranged into 2 symmetric plates of 30 neuroblasts (NB), one median NB (MNB), and seven midline precursors (MP) (Bate, 1976; Bate and Grunewald, 1981)

(ii) Each neuronal precursor can be identified by its position in the neuroepithelium. Some can be identified by the family of neurones they produce (Raper, Bastiani and Goodman, 1983; Goodman and Bate, 1981; Goodman and Spitzer, 1979).

(iii) Each NE divides a number of times to give rise to a chain of ganglion mother cells. Each of these divides once to produce two ganglion cells which differentiate into neurones (Goodman and Bate, 1981)

(iv) Each NB produces a specific number of progeny with specific identities (Raper et al., 1983, Goodman and Bate, 1981; Goodman and Spitzer, 1979).

(v) Each NB develops from a restricted group of cells that share a common potential. Cell-cell interactions are important in determining which ectodermal cell becomes a neuroblast (Taghert, Doe and Goodman, 1984).

(vi) Once a NB begins to divide its lineage determines which neurone family it produces ( Taghert and Goodman, 1984).

(vii) Certain neurones play a "pioneering" role in establishing the basic plan of tracts within the central nervous system. At this early stage axons need only cover small distances by simple and direct routes (Bate and Grunewald, 1981; Goodman, O'Shea, McCaman and Spitzer, 1981).

(viii) Although the segmental ganglia are distinctly different in the adult, both in the number of neurones and in the behavioural functions associated with them, the precursor cells are identical. This is probably achieved by the lifespan of NBs being different in different ganglia and to the fact that large numbers of neurones die as well, many after they have differentiated and produced axons. (Bate, 1976; Whittington, Bate, Seifert, Ridge and Goodman, 1982).

(ix) The final differentiation of each neurone depends on its mitotic ancestry and the interactions it has with other neurones in its environment (Bastiani and Goodman, 1984).

Once the neurones have developed from the precursor cells they then send out axons which make very specific connections. How these growing neurons can choose the

correct pathway and recognize the specific target cells is unknown. Again the insect is providing extremely useful in elucidation of this problem.

Thomas and Wyman (1982, 1983) decided to tackle the problem by isolating mutations in which the proper neuronal connectivity has been disrupted. They used the fruit fly, Drosophila melanogaster, the molecular biology of which has been studied intensely and is now very advanced. Drosophila exhibit an escape response to a light-off stimulus. The neural network involved, called the giant fibre system has been well studied, both anatomically (see for example Koto, Tanouye, Ferrus, Thomas and Wyman, 1981; King and Wyman, 1980), and electrophysiologically (Thomas and Wyman, 1983; Tanouye and Wyman, 1980). Single-gene mutations were induced in the flies. And then using behavioural screening followed by physiological tests, mutants were isolated in which the synaptic connection between the descending neurone from the brain (GF) and the motoneurone TTM (one of the connections in the giant fibre system) was absent (Thomas and Wyman, 1982; Thomas and Wyman 1983). The mutations were known to be on the X-chromosome. Hence genes have been identified whose proper function is necessary for normal neuronal connectivity. The next step will be to discover how these genes contribute to the development of appropriate connections. For example, do these genes code for surface-bound molecules which act as recognition signals? The molecular biology of Drosophila will allow these genes to be studied at the molecular level, with the ultimate aim being to isolate their products.

By understanding the development of the nervous system in simple animals mechanisms are discovered that could be used in more complex ones. Many more additional factors may be involved but at least possible starting points for research are provided. Often it is knowing exactly where to start that is one of the most difficult decisions, since no-one wants to begin research that could lead them nowhere.

Another area where insects are providing a lot of information is in the neural basis of behaviour. The locust is the insect most frequently used, with its flight, jumping and walking systems being those most intensively studied.

#### LOCUST FLIGHT

A locust has two pairs of wings, the forewings and the hindwings, which are attached, respectively, to the meso- and the metathoracic body segments. They are moved by two sets of wing muscles which are located in the same segments. It has been shown that: (i) the basic flight rhythm of the locust is generated primarily at the interneuronal level; (ii) spiking pre-motor interneurons are largely responsible for driving the motoneurons; (iii) the flight oscillator is not continuously active; and (iv) the motoneurons are driven by homologous sets of interneurons as well as other interneurons (Robertson and Pearson, 1982, 1983). It is also thought that the flight rhythm generator is a single entity distributed among several segmental ganglia since individual interneurons that cause the movement of both the hindwing and the forewing have been found (Robertson and

Pearson, 1983, 1984). Some other of the interneurons that have been studied were shown to affect the timing of the flight rhythm (Robertson and Pearson 1982, 1983, 1984). These interneurons were only active once flight was in progress. These are the neurons that could be used in flight manouvers since most of these involve changing the phase of the muscle activity and not the wingbeat frequency (Simmons, 1980). Interestingly, sets of serial homologues were found in the metathoracic and the first three abdominal ganglia (Robertson and Pearson, 1982, 1983, 1984). No wing musculature is located in the abdominal ganglia and so it has been suggested that this reflects the evolutionary history of the wing since they are thought to have originated from appendages which had homologues on all the abdominal segments (Robertson, Pearson and Reichert, 1982). Robertson and Pearson (1984) concluded, therefore, that interneuronal systems may involve some redundancy and so it may not always be easy to explain the functions of some neurons on the basis of good efficient design.

### THE LOCUST JUMP

Spiking interneurons have also been found to be key elements in the neural circuit for the locust jump and kick (Pflüger and Burrows in 1978 showed that the basic motor pattern was the same for these). The jump consists of three phases (Heitler and Burrows 1977; Pearson and Robertson 1981).

(i) Cocking: an initial flexion which brings the tibia into the pre-movement position.

(ii) Co-contraction: simultaneous contraction of the hind

leg flexor and extensor muscles. The energy is stored in elastic elements of the legs and muscles.

(iii) Triggering: The flexor muscles relax, allowing the tibia to extend.

Cocking is brought about by a pair of identified thoracic interneurons (C-neurons). These co-activate the flexor and extensor motoneurons. Co-contraction is maintained by peripheral feedback to the extensor and flexor motoneurons and by an excitatory pathway from the extensors to the flexors. Triggering is brought about by another pair of identified interneurons (M-neurons). These inhibit the flexor extensor motoneurons, the flexors relax and the tibia extend (Heitler, 1983; Pearson, 1983; Pearson and Robertson, 1981).

The C- and M-neurons receive a multimodal sensory input (Pearson, 1983). The same is also thought to occur with some of the interneurons involved in the flight system (Rowell and Pearson, 1983). So we have the interneurons assimilating and gating much of the the sensory input, which results in the production of the appropriate motoneurone activity and behavioural response.

### LOCUST WALKING

Locomotion in arthropods and vertebrates is brought about by reciprocal movement of limbs. This results from appropriate sequences of spikes in sets of motoneurons to the participating muscles. Little is known, however, about the higher order neurons that drive the movements. In insects only a few motoneurons innervate a muscle and these



motoneurones have few connections between them. Hence the activation of the appropriate motoneurones is the responsibility of interneurones (Burrows, 1980). In the locust, local non-spiking interneurones are thought to play a major role in controlling walking and posture (Pearson and Fourtner 1975; Wilson and Phillips, 1982). Some of these interneurones have been found to be excitatory, others inhibitory. Some synapse with one motoneurone, others with several. In the latter case, if certain of the motoneurones drive antagonistic muscles, then the synapses are sometimes reciprocal (Burrows, 1980). Other interneurones have been found which increase the frequency of motor spikes when they are depolarized, and decrease the frequency when they are hyperpolarized (Burrows, 1980; Wilson and Phillips, 1982). All the non-spiking interneurones release transmitter in a graded fashion (Burrows, 1979), with the afore mentioned group being tonically active at their resting potential (Burrows, 1980; Wilson and Phillips, 1982). Tonic graded control will allow slight changes in the relative tension of muscles and hence allow movements to be much more carefully and efficiently controlled than would be achieved by spiking, all or none interneurones.

From the above it can be seen that certain types of neurones play key roles in the neuronal circuits used to generate movement in the locust. It could also be that these same types of neurone perform similar functions in vertebrates. At least these results again provide starting points from which ideas and hypotheses can be formed and tested.

INSECTS - THE PHARMACOLOGY AND ELECTROPHYSIOLOGY OF THEIR  
CENTRAL NERVOUS SYSTEM SYNAPSES

Even though the insect nervous system is relatively simple and it is possible to work with single identified neurones, conclusive prove of a neurotransmitter role for any substance in the CNS is still lacking. For the proof to be conclusive a number of criteria have to be met as follows:

- (a) The substance must be shown to be present within the presynaptic terminals.
  - (b) It must be shown to be released when the presynaptic terminal is stimulated.
  - (c) The necessary synthesizing enzymes must be present within the presynaptic neurone.
  - (d) The appropriate mechanism for inactivation of the substance must be present near the synapse. This may mean the identification of: (a) enzymes for degradation of the substance (as in the case of acetylcholine): or (b) a specific uptake mechanism (as in the case of the biogenic amines).
  - (e) The actions of the natural transmitter must be mimicked on application of the substance at the synapse.
  - (f) The postsynaptic affects of the natural transmitter and the putative one must be identically affected by drugs.
- It must be said that to satisfy all these criteria would require very favourable conditions, and in fact it is worth noting that for many putative transmitters only one or two have been met.

In the insect the putative central transmitters which have



received the most attention are acetylcholine (ACh), the biogenic amines dopamine (DA), noradrenaline (NA), octopamine (OCT) and 5-hydroxytryptamine (5-HT) and the amino acids gamma-amino butyric acid (GABA) and glutamate (Glu). In the following sections these compounds will be considered in turn, and the evidence for their role as a neurotransmitter presented.

Before tackling this, however, it should be mentioned that a large number of substances have in fact been identified in insect neurones, although for most no idea is known about their possible function. One reason for this is that it is only in the last few years that many of them have been shown to be present, due to the development of the technique of immunocytochemistry (see Pitman, 1984, for an extensive list). The main drawbacks with this are that only a few of the neuropeptides have been isolated and characterized in insects and even fewer are available in sufficient amounts to allow antibody production. Hence, in many cases vertebrate antibodies have been used, which brings with it the possibility of non-specific staining and cross-reactivity with unknown antigens. And so unless confirmed by other methods, the immunoreactive compound must remain chemically unidentified.

There are a few exceptions to the above, the pentapeptide, proctolin, being the most well known. This peptide was isolated from the cockroach hindgut, purified, sequenced and after physiological experiments proposed as the neuromuscular transmitter of motoneurones that innervate hindgut

muscles (Brown and Starrat, 1975; Starrat and Brown, 1975; Eckert, Agricola and Penzlin, 1981). More recent evidence has shown that it also has a role at the insect skeletal neuromuscular junction (Bishop, O'Shea and Miller, 1981; O'Shea and Adams, 1981; Adams and O'Shea, 1983; O'Shea, 1985). It has been found to be a co-transmitter along with glutamate in the slow coxal motoneurone (Ds) of the cockroach and in the slow extensor motoneurone (SETi) of the locust extensor tibiae muscle. Stimulation of Ds or SETi produces two responses, the first being a glutamate-mediated transient contraction. For Ds, the second is a proctolin dependent slow and sustained rise in tension; and for SETi, it is a proctolin dependent stimulation of the muscle's myogenic rhythm.

Several other peptides have also been found to accelerate the myogenic rhythm. Locust adipokinetic hormone (AKH) and the structurally similar myogenic factors, MI and MII (isolated from cockroach corpus cardiacum), all produce an increase in myogenic frequency and a tonic contraction of the muscle. Both of these actions are reminiscent of proctolin, although the increased myogenic frequency for the AKH-related peptides is longer lasting (O'Shea, Witten and Schaffer, 1984; O'Shea and Schaffer, 1985; O'Shea, 1985).

Finally, FMRFamide-like peptides are another family whose action at the locust extensor tibiae has recently been investigated. As with the above mentioned peptides they can bring about an increase in basal tension in the muscle. They do not, however, affect the myogenic rhythm but instead

can modulate SETi-induced twitch tension (Walther, Schiebe and Voigt, 1985; Evans and Myers, 1986).

ACETYLCHOLINE

This section presents a review of the evidence to date for the role of ACh as neurotransmitter in the insect CNS. The evidence is discussed under five sub-headings and in general each is dealt with in a chronological manner, beginning with the earliest work.

There is more evidence to support the role of ACh as a neurotransmitter in the insect central nervous system (CNS), especially at sensory nerve terminals, than for any other of the candidates. One of the reasons for this is that historically, ACh was one of the first substances to be studied in the insect CNS, where it was in fact shown to be present in relatively large amounts. Table I.1 shows the values that have been reported for the CNS of the cockroach. These values are in fact higher than those obtained for mammalian sympathetic ganglia (Brown and Felberg, 1936), which are believed to have a largely cholinergic input. Another reason is that the vertebrate cholinergic system has also been studied extensively and so the knowledge gained from this, and the various agonists and antagonists developed for it, have aided the insect studies.

The Distribution of ACh and its Metabolic Enzymes.

From Table I.1 it is seen that ACh is widely distributed throughout the insect CNS, with the concentrations being higher in the ganglia than in the interganglionic connectives (Tobias, Kollros and Savit, 1946; Colhoun, 1958a). ACh was postulated as a possible transmitter from several early investigations, although problems were raised

TABLE I.1

DISTRIBUTION OF ACETYLCHOLINE (ACh) AND ACTIVITIES OF  
ACETYLCHOLINESTERASE (AChE) AND CHOLINE ACETYLTRANSFERASE  
(ChAc) IN THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH  
PERIPLANETA AMERICANA

<u>Tissue</u>	<u>ACh</u>	<u>AChE</u>	<u>ChAc</u>
Nerve cord	64(1,2) 70(5) 32(6)	270(4) 200(7)	11(3)
Brain	144(1,2)	138(4)	51(3)
Brain + sub- oesophageal ganglion	135(1)	138(4)	53(3)
Thoracic nerve cord	80(1,2) 46(8)	221(4) 320(8)	11(3) 0.008(8)
Thoracic ganglia	95(1) 58(8)	332(4)	21(3)
Thoracic connectives	31(1) 34(8)	239(4)	2.6(3)
Abdominal nerve cord	65(1)	188(4)	6(3)
6th abdominal ganglion	63(1)	315(4)	18(3)

ACh expressed in  $\mu\text{g/g}$  tissue wet weight; AChE activity expressed in  $\text{mg ACh hydrolyzed/g tissue/hr}$ ; ChAc activity expressed in  $\text{mg ACh synthesized/g tissue/hr}$ .

REFERENCES

- |                    |                                     |
|--------------------|-------------------------------------|
| 1. Colhoun (1958a) | 5. Mikalanos and Brown (1941)       |
| 2. Colhoun (1958b) | 6. Roeder (1948)                    |
| 3. Colhoun (1958c) | 7. Tobias, Kollros and Savit (1946) |
| 4. Colhoun (1959)  | 8. Yamasaki and Narahashi (1960)    |

due to its very low potency (Roeder, Kennedy and Samson, 1947; Roeder, 1948; Hopf, 1952; Twarog and Roeder, 1956, 1957; Harlow, 1958; Yamasaki and Narahashi, 1958, 1960; Vereshchagin, Sytinsky and Tyshchenko, 1960; Suga and Katsuki, 1961). There appear to be two reasons for this. Firstly, insects have a well developed blood-brain barrier system, which is due to the intercellular occlusions at the ends of the perineural clefts. The main restriction is to water soluble substances (see Treherne and Pichon, 1972). Secondly, the ACh is rapidly hydrolyzed by cholinesterases which are associated with the glial cells that surround the neurones (Treherne and Smith, 1965a, 1965b; Lord, Gregory and Burt, 1967). This means that ACh has only very restricted access to the receptors, which results in the requirement of high doses. To increase the access, removal of the nerve sheath from the ganglia and treatment with the acetylcholinesterase inhibitor, eserine, is necessary. In 1958 and 1960 Yamaski and Narahashi, studying transmission between the cercal nerves and the giant fibres in the sixth abdominal ganglion of Periplaneta americana, showed that: (a) the threshold for intact ganglia was 10 mM ACh, (b) the threshold was reduced to 1 mM ACh if the ganglia were desheathed or eserinizied, (c) both treatments together further reduced the threshold to 0.1 mM.

With the knowledge of vertebrate cholinergic systems, information was gained about the various elements that are found at a cholinergic synapse (see Cooper, Bloom and Roth, 1978, for a more detailed discussion). It was shown at the vertebrate synapse that:

- (a) ACh is synthesized mainly in the cytoplasm of the presynaptic terminals, from choline and acetyl coenzyme A by the enzyme choline acetyltransferase (ChAc).
- (b) It is taken up into and stored in vesicles, ready for release into the synaptic cleft on activation of the terminal.
- (c) Its postsynaptic action is terminated by hydrolysis to choline and acetate by the enzyme acetylcholinesterase (AChE).
- (d) The choline produced is then returned to the terminal by a high-affinity uptake system, where it can be reused for the production of more ACh.

From the above it is obvious that to show a neurotransmitter role for acetylcholine it is necessary to show the presence of the synthesizing and degrading enzymes in the preparation, preferably at terminal endings. It would also be desirable to show the release of ACh on stimulation of the terminals, although this is difficult to do.

Table I.1 shows that it has been demonstrated that both AChE and ChAc are present in fairly high concentrations and also widely distributed in the insect CNS. ChAc in particular was shown to have a much higher activity in the ganglia than in the connectives (Colhoun, 1958c). This would be expected if ACh synthesis occurs mainly at nerve terminals. In 1941 Mikalonis and Brown found that electrical stimulation of eserinated cockroach ventral nerve cord caused an increase in the amount of ACh released into a perfusion solution. Then Colhoun (1958b) showed that the ACh content of eserinated thoracic nerve cords also increased when the anal



cerci were stimulated or the connectives between the 5th and 6th abdominal ganglia were electrically stimulated. The drawback with these experiments is that results are for whole ganglia. To show a neurotransmitter role for ACh much more specific preparations are required, those which use a particular group of synapses or identified cells and which allow enzyme and terminal release blockers to be used.

Emson, Burrows and Fonnun, 1974, working on identified neurones in two species of locust, were unable to detect any ChAc activity in the cell bodies of both the excitatory and inhibitory motoneurones. They did, however, detect fairly large quantities in the whole thoracic ganglia. It may be a negative result but it does suggest that ChAc must be selectively distributed in the ganglion. They also measured ChAc activity in animals that had undergone leg amputation and found that there was a large fall in the specific activity in the nerve five stump. This operation would have caused the sensory nerve fibres to degenerate, since in general they have peripheral cell bodies, and so this suggests that at least some sensory <sup>r</sup>ve terminals are cholinergic.

The specific distribution of AChE within the insect CNS has been studied several times using histochemical methods (Iyatomi and Kanehisa, 1958; Wigglesworth, 1958; Winton, Metcalf, and Fukuto, 1958; Burt, Gregory, and Molloy, 1966), but it was with an ultrastructural study (Smith and Treherne, 1965) that the most information was gained. They showed that the enzyme was present in the glial cells which



surround the cell bodies, in the glial sheaths which surround the nerve fibres and in restricted areas in the neuropile. This enzyme was eserine-sensitive. They also found eserine-insensitive activity but it is thought that this may not be involved in neuronal transmission since its inhibition did not affect cercal nerve - giant fibre transmission (Colhoun, 1960).

#### ACh Receptors.

More evidence to support ACh as an insect neurotransmitter has come from ligand binding experiments of CNS extracts, which have resulted in the characterization of three putative cholinergic receptors. Using  $\alpha$ -bungarotoxin, a highly specific nicotinic antagonist, a receptor with nicotinic properties has been isolated from the low-speed extract of nervous tissue of a number of insects including Drosophila melanogaster (Schmidt-Nielson, Gepner, Teng and Hall, 1977; Dudai, 1977, 1978; Rudolf 1978), Periplaneta americana (Gepner, Hall and Sattelle, 1978), Musca domestica (Harris, Cattell and Donnellan<sup>1979</sup>), and Manduca sexta (Sanes, Prescott and Hildebrand, 1977). Using the specific muscarinic receptor blocker, quinuclidinyl benzilate, a receptor with muscarinic properties has also been isolated from Drosophila melanogaster slow-speed nervous tissue extract (Haim, Nahum and Dudai, 1979). Finally a receptor with mixed activity has been isolated from high-speed nervous tissue extracts of Musca domestica (Donnellan, Jewess and Cattell, 1975).

ACh as the Transmitter at Sensory Synapses.

Various experiments have provided fairly strong evidence that ACh is the transmitter used by sensory neurons in the antenna of the spinx moth Manduca sexta. This insect, like all Lepidoptera, has only rudimentary antennae and antennal brain centres at the larval stage. These then degenerate at pupal ecdysis and are replaced by much larger structures in the developing adult (Norlander and Edwards, 1970). Also at this time the sensory neurones arise and differentiate in the antennae and send their growing axons into the developing lobes (Sanes and Hildebrand, 1976a). Using radiochemical screening procedures to assay for ACh and ChAc, and a colourimetric procedure to assay for AChE, Sanes and Hildebrand (1976b) showed that developing adult antennae contain ACh and the two enzymes. These are first detected not long after the sensory neurones are formed and appear within the head soon after the arrival of the antennal fibres. Using the same detection methods Prescott, Hildebrand, Sanes and Jewett (1977) measured ACh and its two enzymes at various times during postembryonic development. There is a dramatic rise (more than a 1000-fold) in ChAc activity and a large increase in ACh stores in the antennal lobes, both of which parallel the arrival of the sensory axons. The AChE profile is slightly different, but this is probably due to the fact that this enzyme is also associated with ganglionic neurones and glia, the developmental timetable of these not being identical to that of the sensory neurones. If the developing antennal lobe is removed before the antennal fibres make contact, it is found that the

fibres still contain ACh, ChAc and ChE (Sanes, Hildebrand and Prescott, 1976), indicating that these neurones can still develop without their target cells. However, if the lobes are deafferented by amputation of the developing antennae the levels of ACh and the two enzymes are greatly reduced. This was not the case, however, with the level of  $\alpha$ -bungarotoxin binding activity, which stayed nearly normal (Sanes, Prescott and Hildebrand, 1977; Hildebrand, Hall and Osmond, 1979). This binding is presumably linked with postsynaptic ACh receptors and so suggests that the differentiation of neurons in the lobe is independent of normal sensory input.

For the antennal nerve sensory synapse described above most of the evidence has been obtained from various biochemical methods in adult and developing animals. Another way to tackle the problem of identifying a transmitter is pharmacologically, by observing the effects of agonists and antagonists of the putative transmitter on synaptic transmission. In the case of sensory input to the insect CNS this type of approach has largely centred on the cercal nerve - giant interneurone synapse of the cockroach Periplaneta americana.

#### Electrophysiological Evidence.

In the cockroach ventral nerve cord there are about seven pairs of giant interneurons with cell bodies in the sixth abdominal ganglion. Their axons extend to the brain. It is also in the sixth abdominal ganglion that these neurones synapse with the afferent fibres that originate from the

sensory appendages known as the anal cerci. These synapses form a major input onto the dendritic arborization that the interneurons have in this ganglion. The large size of these interneurons allows their electrical activity to be monitored with relative ease, with the result that much work has been carried out on this preparation.

Early experiments on the giant interneurons used external hook electrodes to monitor the synaptic transmission (see for example, Roeder et al., 1947; Twarog and Roeder, 1957; Yamasaki and Narahashi, 1958, 1960; Shankland, Rose and Donniger, 1971). These early investigations of the action of ACh showed that it had an excitatory effect but only at high doses, with desheathing of the ganglion and eserization causing a marked decrease in the concentration of ACh required to elicit a response (see page 22). Flattum and Shankland (1971), and Shankland et al (1971) reasoned that the response to applied ACh and cholinergic drugs would not only be affected by tissue cholinesterases but also by endogenous transmitter. Hence in their experiments they pretreated the ganglia with hemicholinium-3 (HC-3, which inhibits choline reuptake in mammalian neurones thereby depleting ACh stores), as well as with a potent cholinesterase inhibitor (either dichlorvos or paraoxon or dimethyl 2,2-dichlorovinyl phosphate). This resulted in preparations that were highly sensitive to ACh, with about three quarters of them responding to 1  $\mu$ M ACh.

A variety of cholinergic agonists have been applied to the cockroach sixth abdominal preparation, with the results being as follows. Twarog and Roeder (1957) found that

the muscarinic agonists, muscarine and pilocarpine, when bath applied at concentrations in the range  $1 - 0.1$  mM, evoked asynchronous bursts of spikes in the giant interneurons. Shankland et al (1971) using their pretreated preparations (see above) demonstrated that nicotine and the muscarinic agonist, acetyl- $\beta$ -methylcholine, were excitatory at concentrations as low as 10 nM.

Several cholinergic antagonists have been shown to block transmission at these synapses as follows. Twarog and Roeder (1957) found that hexamethonium, decamethonium, benzoquinonium, tetramethylammonium, and hydroxyphenyltrimethylammonium all blocked transmission. Shankland et al 1971 showed that atropine, a muscarinic antagonist, blocked synaptic transmission at a threshold concentration of  $0.1 \mu\text{M}$  and that the nicotinic antagonists, d-tubocurarine, hexamethonium and decamethonium, blocked transmission at  $0.1 - 1 \mu\text{M}$ ,  $1 \mu\text{M}$  and  $1 \text{ mM}$  respectively. Flattum and Sternburg (1970a, 1970b) found that the preparation's response to nicotine desensitized, with the increase in spontaneous activity which occurs when nicotine is first applied being followed by depression and blockade of synaptic transmission. This was then followed by recovery of both, with synaptic transmission continuing even when nicotine was present. Flattum and Shankland (1971) also found this. Using a preparation which had been desensitized with nicotine and then allowed to recover they found that d-tubocurarine (nicotinic antagonist) had no effect on the activity of the preparation, whereas the muscarinic antagonist, pilocarpine, was more potent. They also found

that desensitized preparations were still able to respond to ACh and acetyl- $\beta$ -methylcholine (muscarinic agonist) and that these responses were blocked by atropine (muscarinic antagonist).

The above results show that nicotinic and muscarinic agonists and antagonists can, respectively, excite the preparation or cause transmission to become blocked. They also demonstrate that muscarinic agonists are still able to elicit a response even in the presence of a desensitizing concentration of nicotine. Taken together these results suggest that the cockroach giant interneurons may possess both nicotinic and muscarinic receptors rather than a single class of receptors with a broad pharmacological profile. It must be noted, however, that other neurons will synapse with the giant interneurons. If they also have cholinergic receptors, they too will be affected by cholinergic agents and this could indirectly affect the giant interneurons.

More recently two new methods of extracellular recording have been introduced for the giant interneurone preparation. These are the mannitol (or sucrose) gap technique (Callec and Sattelle, 1973; Sattelle, McClay, Dowson and Callec, 1976; Callec, Sattelle, Hue and Pelhate, 1980), and the oil gap technique (Callec and Boistel, 1971c; Callec 1974). With both these methods it is possible to monitor synaptic EPSPs and IPSPs with nearly the same accuracy as intracellular electrodes. It is only the oil gap technique, however, that allows experiments on single identified neurons to be carried out. Using the mannitol gap method the sequence of effectiveness of certain cholinergic agonists was found to



be nicotine > ACh (+ eserine) > carbachol > pilocarpine > ACh. For the antagonists the order was found to be  $\alpha$ -bungarotoxin > d-tubocurarine > benzoquinonium > hexamethonium = atropine (Callec et al., 1980, Sattelle, 1980). With the oil gap technique extracellular recording is made from a single fibre. Hence it is possible to apply drugs iontophoretically onto the cell's dendritic field and by so doing circumvent the peripheral cholinesterase barrier. Using this approach it has been found that ACh causes a depolarization of the neurone and a decrease in its membrane resistance (Callec and Boistel, 1971c). They also monitored the size of both electrically induced EPSPs and the acetylcholine-induced response. It was found that hyperpolarization caused both responses to increase in size, depolarization caused them to decrease, and that their reversal potentials were similar. This was estimated to be about -35 mV by Callec (1974). These data suggest that a similar ionic mechanism underlies both responses.

In 1980 Harrow, Hue, Pelhate and Sattelle developed a single-axon back-filling technique, that allowed individual staining of the giant interneurons. By combining this neuroanatomical method with the oil gap single-fibre technique they were thus able to study single identified neurones. In this way they demonstrated that bath applied  $\alpha$ -bungarotoxin (10 nM) irreversibly blocked unitary EPSPs evoked in two identified interneurons by deflection of a single cercal mechanoreceptor (Harrow, Hue, Pelhate and Sattelle, 1979; Sattelle, Harrow, Hue, Gepner and Hall, 1983). Harrow and Sattelle (1983) found that  $\alpha$ -bungarotoxin

also blocked the ACh response of one of the interneurons mentioned above. The muscarinic antagonist, quinuclidinyl benzilate, was ineffective.

#### ACh as a Transmitter at other Central Synapses.

The pharmacological approach has been used fairly extensively to investigate the possible role of ACh as a transmitter in only one other group of insect CNS neurones. These are the dorsal unpaired median neurones (DUM) which are found in clusters around the dorsal midline of a number of insect species. The responses are monitored intracellularly from the neurone somata. The caution here is that although receptors are found on the cell bodies, no synapses are. It is not known if these receptors are identical to the synaptic ones and so it is possible that a drug may have different effects at the two sites.

Intracellular recordings from DUM neurones have shown that they usually have a resting potential of around -55 mV and that overshooting action potentials as well as EPSPs and IPSPs can be recorded (Callec and Boistel, 1966, 1967; Kerkut, Pitman and Walker, 1969a, 1969b). Also, by intracellular injection of Procion Yellow, it was shown that the cells have symmetrical bifurcating axons which in some cases extend into the peripheral nerves (Crossman, Kerkut, Pitman and Walker, 1970; Hoyle, Dagan, Moberly and Colquhoun, 1974).

In 1967 Callec and Boistel found that locally applied, pressure ejected, ACh (600 - 60  $\mu$ M) caused a membrane



depolarization and increased action potential activity. This action of ACh was blocked by pretreatment of the preparation with 100  $\mu$ M gallamine. Slightly later it was shown that iontophoretically applied ACh gave a transient depolarization. It was also found that both this response and the EPSP evoked by stimulating the right anterior connective were increased in amplitude on hyperpolarization of the neurone (Kerkut et al., 1969a, 1969b; Pitman and Kerkut, 1970). By extrapolating plots of response amplitude versus membrane potential, Pitman and Kerkut (1970) estimated that the reversal potential of the ACh response was  $-45.3 \pm 3.1$  mV, whereas that of the EPSP was  $-40.3 \pm 1.6$  mV. They also showed that gallamine (400  $\mu$ g/ml) reversibly blocks both responses, whereas eserine (10  $\mu$ g/ml) increases the the amplitude and duration of both. The similarity of the reversal potentials suggests that both phenomena have the same ionic mechanism. The similarity of the action of gallamine and eserine suggest that the natural transmitter at some of the DUM neurone synapses could possibly be ACh.

From the experiments using bath applied ACh it was found that high concentrations were required to elicit a response and this caused problems when considering ACh as a transmitter candidate. By applying ACh locally using an iontophoretic electrode Kerkut et al (1969a, 1969b) showed that the cells were in fact quite sensitive to ACh. By extrapolation from a depolarization amplitude - iontophoretic current plot they calculated that as little as 0.1 pM ACh was required to produce a response. Kerkut et al (1969b) and Kerkut, Newton, Pitman, Walker and Woodruff

(1970) studied the effects of various cholinergic ligands. They obtained the following order of potency for cholinergic agonists: nicotine > carbamoylcholine > muscarone > furtrethonium > McN-A-343 > pilocarpine = acetyl- $\beta$ -methylcholine > ACh. From this it is seen that nicotinic agonists are more potent than muscarinic ones. However, the low potency of ACh was due to its rapid hydrolysis by tissue cholinesterases and this could possibly have been the same for acetyl- $\beta$ -methylcholine. Hence the rank order may not be the true one. These workers also found that d-tubocurarine (100  $\mu$ g/ml) inhibited the activity of the preparation and also antagonized the response to nicotine. Atropine (10  $\mu$ g/ml) antagonized the responses to ACh and pilocarpine. All these results support the view that these neurones possess cholinergic receptors.

More recently Goodman and Spitzer (1979, 1980) have examined the sensitivity of embryonic DUM neurones in the grasshopper (Schistocerca nitens) to bath application and iontophoresis of various cholinergic agents. They found that at day seven no neurones responded to ACh but by day thirteen several of the neurones gave a depolarizing response, to both bath applied and iontophoretic ACh. They calculated that the reversal potential for the ACh response was +20 mV (but cf. value of about -45 mV obtained by Pitman and Kerkut in 1970) and found that the response was abolished by removal of sodium ions from the saline. This latter result was also obtained by Pitman and Kerkut (1970). These results suggest that although in both cases sodium ions have a role in the ACh response, the relative

involvement of sodium is different. They also found that nicotine was a potent agonist and that the ACh response was reduced by the nicotinic antagonists curare (0.1 mM) hexamethonium (1 mM) and decamethonium (0.1 mM). Pilocarpine (a muscarinic agonist), however, was ineffective, as were the muscarinic antagonists atropine and quinuclidinyl benzilate at blocking the ACh response. These results suggest that the ACh receptor is primarily nicotinic in nature. But they also found that the response to ACh was not blocked by the usually very effective nicotinic antagonist,  $\alpha$ -bungarotoxin. Sattelle, David, Harrow and Hue (1980) have obtained evidence that the DUM neurones in the metathoracic ganglion of adult cockroaches are also relatively insensitive to  $\alpha$ -bungarotoxin. Obviously more experiments are required to determine whether the observed differences between the embryonic grasshopper neurones and the adult cockroach neurones are due to species or developmental differences. However, the results do suggest that these ACh receptors are different from the ones found at the giant interneurone synapses.

Other insect preparations have been studied electrophysiologically but to a much lesser degree than the two discussed above. A brief summary of the different preparations and their main results is given below.

A few studies have been carried out on the fast coxal depressor motoneurone of the metathoracic ganglion of the cockroach Periplaneta americana. David and Pitman (1979, 1982) found that bath applied and iontophoretically applied ACh depolarized the cell and that repeated application

produced desensitization of the response. Anti-cholinesterases produced up to a 1000-fold potentiation of the normal ACh response. Carbamoylcholine also gave a depolarization. They estimated the ACh reversal potential to be -35 mV. More recently David and Sattelle (1984) obtained the following order of potency for cholinergic agonists: nicotine > ACh + neostigmine > carbamoylcholine > tetramethylammonium. Other agonists (nicotinic and muscarinic) were found to be ineffective. In general nicotinic antagonists were found to be the most effective at blocking the ACh response.

Carr and Fourtner (1980) studied the pharmacological properties of the cockroach "slow" coxal depressor motoneurone, which makes monosynaptic connections with trochanteral hair plate sensory neurones. Synaptic transmission was blocked by a number of cholinergic antagonists which suggests that ACh may be the transmitter. In 1961 Suga and Katsuki provided evidence that ACh may also be one of the transmitters used at auditory synapses in the grasshopper, Grampsocleis buergeri. Two new approaches to insect pharmacology were developed by Usherwood, Giles, and Suter (1980). The first technique involved culturing insect neurones from embryos, nymphs and adults; the second involved the isolation of neurones from the adult CNS. The pharmacology of the dissociated neurones and the nymphal cultured neurones were similar. ACh caused a depolarization which was antagonized by both atropine and d-tubocurarine. These results indicate the presence of cholinergic receptors. But it is not known whether there is one receptor

type with a broad pharmacological specificity, or if there is a mixed nicotinic-muscarinic receptor population.

BIOGENIC AMINES

For some time now, lists of putative neurotransmitters and neuromodulators in the CNS of both vertebrates and invertebrates have always included the catecholamines, dopamine (DA) and noradrenaline (NA), and the indolalkylamine, 5-hydroxytryptamine (serotonin or 5-HT). More recently the phenolamine, octopamine (OA), has been added to the invertebrate list. Very little is known, however, about the functional roles played by these amines in the insect CNS. One of the main reasons for this, is that the work carried out to locate amine containing neurones has been concentrated on the areas of the CNS that are fairly inaccessible to electrophysiological and pharmacological techniques. In the last few years this trend has begun to change, and so it is hoped that a discovery of some of the roles of amines will soon be forthcoming.

The Location of the Biogenic Amines.

In the early experiments to ascertain the quantities of amines present, fluorescence-based assays of whole insect homogenates were used. These assays were relatively insensitive but did show the presence of NA and DA. Disagreement arose, however, as to whether adrenaline was present or not (Ostlund, 1953; von Euler, 1961). These results in fact tell us very little since it is now known that amine derivatives are also involved in cuticular tanning (Karlson and Sekeris, 1962), and so some of the amines detected may have no neural function. Since then



much more sensitive fluorescence-based assays, and more recently developed radioenzymatic techniques, have allowed fairly accurate quantitative measurements of biogenic amines in various parts of the insect CNS, and even in identified single cells (see for example Frontali and Häggendal, 1969; Kusch, 1975; Dymond and Evans, 1979; Evans and O'Shea, 1978). Table I.2 gives values obtained for the cockroach, Periplaneta americana. In general, the amounts of DA, 5-HT and OA are of the same order of magnitude, with the content of NA being about ten times lower. Only three groups of workers have shown the presence of adrenaline: Amarti and Gilmour (1976) working on the Queensland fruit fly, Dacus tryoni; and Kostowski, Tarchalska-Kyrnska and Marowska (1975) and Kostowski, Tarchalska and Wanchowiz (1975), working on the ant, Formica rufa. It is not known whether these results represent a genuine difference for these two species or whether the techniques used were not specific for adrenaline.

In 1965 the advent of the Falck-Hillarp technique (see Falck and Owman, 1965) allowed the location of DA, NA and 5-HT in the insect CNS to be determined. Since then studies on a wide range of insect species have been carried out, and many fluorescent neurones and fibre bundles have been seen (see Klemm, 1976, for an extensive review). This work, however, has mainly concentrated on the brain where the anatomy is complex and where the neurones are frequently small. Hence it is very difficult to try to identify the functions of the amine-containing neurones and the result is that little progress has been made. The thoracic and abdominal ganglia

TABLE I.2

DISTRIBUTION OF THE BIOGENIC AMINES IN THE CENTRAL NERVOUS  
SYSTEM OF THE COCKROACH PERIPLANETA AMERICANA

<u>STRUCTURE</u>	<u>NA</u>	<u>DA</u>	<u>OA</u>	<u>5HT</u>
Optic lobes (per pair)	0.36(1)*	2.41(1)*	7.24(2)*	
Supraoesophageal ganglion	5.53(1)*	6.98(4)\$ 26.81(1)*	4.68(2)*	12.27(4)\$
Supra- + sub-oesophageal ganglia	2.19(3)\$	16.32(3)\$		
Suboesophageal ganglion	0.33(1)*	3.29(1)*	5.60(2)*	
Thoracic nerve cord		8.27(1)*		
Prothoracic ganglion	0.29(1)*	2.61(1)* 3.20(4)\$	5.28(2)*	11.70(4)\$
Mesothoracic ganglion	0.23(1)*	2.28(1)* 2.28(4)*	4.33(2)*	8.33(4)\$
Metathoracic ganglion	0.16(1)*	2.14(1)* 1.18(4)\$	5.32(2)*	9.02(4)*
Abdominal nerve cord		6.69(1)*		
Sixth abdominal ganglion	0.05(1)*	1.53(1)* 2.02(4)\$	5.26(2)*	15.41(4)\$

\* = Values expressed as pmoles amine/ structure.

\$ = Values expressed as nmoles amine/ g tissue wet weight.

REFERENCES

1. Dymond and Evans (1979)
2. Evans (1978a)
3. Frontali and Häggendal (1969)
4. Kusch (1975)



are much more amenable to electrophysiological techniques; the layout of these ganglia has been well studied and the neurones are much more accessible, allowing the possibility of working with identified cells. Until very recently, however, there has been little success in location of amine-containing neurones in these ganglia. The only real exception has been the location of dopamine-containing cell bodies in the ventral nerve cord of Trichoptera (Björklund, Falck and Klemm, 1970; Klemm, 1971). Unfortunately, the cell body positions did tend to be very variable. It was in 1981 that a breakthrough was made with Viellemaire, Cailley-Lescure, Bensch and Girardie locating the positions of several catecholamine- and serotonin-containing neurones the suboesophageal and ventral nerve cord ganglia of the locust using the Falck-Hillarp technique. Then in 1983 Bishop and O'Shea used immunocytochemistry to locate the positions of serotonin-containing neurones in the same ganglia of the cockroach. In both cases, some of the neurones were fairly large and so it should be possible to carry out the experiments necessary to ascertain the function of these cells.

Octopamine is the other biogenic amine that has been proposed as a transmitter candidate but it cannot be located by histochemical means. In 1974 Hoyle, Dagan, Moberly and Colquhoun had found that certain of the dorsal unpaired median (DUM) neurones in the locust and grasshopper metathoracic ganglion innervated the major skeletal muscles of the hindleg, and that the nerve endings were of the neurosecretory type. The interganglionic morphology and

electrophysiology of these cells had been studied a few years previously (Kerkut et al., 1969b; Crossman et al., 1971). Hoyle et al (1974) focused on one neurone in particular which innervated the extensor tibiae (ETi) muscle, and called it DUMETi. In 1975 Hoyle showed that stimulation of DUMETi inhibited the myogenic rhythm of the hindleg extensor tibiae muscle and that this effect could be mimicked by the infusion of saline containing low concentrations of DA (1  $\mu$ M), NA (1  $\mu$ M), and OA (2.5 nM). None of the DUM neurones fluoresced after the Falck-Hillarp technique, and so he suggested that they must contain OA. In confirmation of this Hoyle and Barker (1975) showed that DUM neurones could synthesize OA from tritiated tyrosine. More recently Evans and O'Shea (1977, 1978) isolated physiologically identified DUMETi neurones and showed that they each contain about 0.1 pmol of octopamine, at least 800 times the concentration found in identified motoneurones. Dymond and Evans (1979) have also shown that other DUM neurones contain an equivalent amount of OA as DUMETi. The peripheral action of this neurone on muscle has been extensively studied (Evans and O'Shea, 1977, 1978; O'Shea and Evans, 1979; Evans, 1981, 1984a, 1984b). Recent work by Sombati and Hoyle (1984a and b) has begun to elucidate the central functions of DUM neurones and this is discussed in pps 41a-c.

As mentioned in the section dealing with ACh, one of the criteria for a neurotransmitter role for a substance is to show the presence of the appropriate metabolic enzymes. As yet no biogenic amine synthesizing or inactivating enzymes

Recent work by Sombati and Hoyle (1984a and b) has started to elucidate the function of DUM neurones in the central nervous system of the insect. These authors found that regardless of which DUM neurone they recorded from in the locust metathoracic ganglion, action potentials were seen just before the start of any leg movements. This occurred whether the movements were spontaneous or evoked (Sombati and Hoyle, 1984a). It had previously been shown that stimulation of the locust fast extensor tibiae motoneurone (FETi) produces EPSPs in the antagonistic flexor motoneurones (Hoyle and Burrows, 1973). During repetitive stimulation this FETi-evoked response progressively declines (Sombati and Hoyle, 1984a). Sombati and Hoyle (1984a) found, however, that this habituation was reversed by iontophoretic application of octopamine into the neuropilar region of presumed synaptic action at the flexor motoneurones, or by concurrent stimulation of a DUM neurone (presumed to be octopaminergic). When larger amounts of octopamine were iontophoresed the EPSP size recorded in the flexor motoneurones increased and in some instances action potentials were initiated. In a further series of experiments Sombati and Hoyle (1984b) showed that iontophoretic application of octopamine at a few specific regions in the neuropile of the metathoracic ganglion caused repetitive bouts of either stepping movements or flight motor activity. Hence from these results it appears that octopaminergic neurones may have at least two roles in the insect central nervous system. They may be involved in the activation of neural circuits which produce specific

behaviours and also in the potentiation of synaptic transmission and hence help to maintain certain motor outputs. In the light of these results Sombati and Hoyle (1984b) suggested the 'Orchestration Hypothesis' which proposes that the excitation of the appropriate modulatory neurones is a prerequisite to the selection, production and maintenance of specific behaviours.

One other study has recently investigated the morphology and ultrastructure of DUM neurones in the locust metathoracic ganglion at the light and electron microscopic level (Watson, 1984). This work concentrated on the DUM neurones which send axons to the muscles; no examination was carried out on the DUM neurones that are interneurones. At the light level it is seen that a single primary neurite arises from the soma and then splits into two lateral neurites which pass into peripheral nerves on each side of the body. Each of these three main neurites gives off secondary branches. At the electron microscopic level Watson (1984) found presynaptic inputs of several types onto the spines of the lateral neurites and onto the secondary branches of DUM neurones. Very few output synapses were observed and they were found only on lateral neurite spines. Watson (1984) therefore concluded that the DUM neurones examined did not play a major role in the CNS.

Hence there appears to be a conflict between the results of Watson (1984) and those of Sombati and Hoyle (1984a and b). It may be that although the number of central output synapses a DUM neurone makes is small, each one is very

potent, or that it is the interneuronal DUM cells which have the major central actions. Only further experiments will clear up this problem

have been isolated from insect nervous tissue. The metabolic pathways for handling the biogenic amines are, however, well understood in mammals. And so most studies on the insect has involved comparing the results of labelled precursor incubation experiments with the mammalian pathways and thereby deducing the presence or absence of certain enzymes. Care must be taken with this, however, due to the non-neural function of amines in tanning. It is known that N-acetyl dopamine acts as a protein cross-linking agent in the formation of sclerotin, and that cuticle formation occurs in the haemolymph (Whitehead, 1969), but the location of the N-acetylation step is still uncertain. What really is required is demonstration of the various amines and enzymes in specific locations.

From the work so far it does appear that OA and DA are synthesized from tyrosine, and 5-HT from tryptophan, probably by the same pathways as in mammals (Hoyle and Barker, 1975; Maxwell Tait and Hildebrand, 1978; Osborne and Neuhoff, 1974b). As yet no synthesis of NA has been demonstrated, but it is present in much smaller amounts. It is also uncertain whether or not the first step in the conversion of tyrosine to DA is decarboxylation (see Vaughan and Neuhoff, 1976; Mir and Vaughan, 1981) rather than hydroxylation (as is found in mammals). Finally, it is still not known if the decarboxylation of the catecholamine and 5-HT precursors is carried out by the same enzyme or not.

The postsynaptic effects of biogenic amines in mammals are terminated by reuptake mechanisms. The amines are then

reused, if taken up into the presynaptic terminals, or enzymatically inactivated, if taken up into other neuronal or non-neuronal elements. In 1978b Evans described a three component uptake mechanism for OA in the ventral nerve cord of the cockroach, Periplaneta americana. The system consisted of high and low affinity sodium sensitive components and a sodium insensitive component. Experiments with agonists and antagonists showed that the high affinity sodium sensitive component is similar to the one found in mammalian heart (Iversen, 1965; Evans, 1978b). This does suggest that amines in insects are dealt with in a similar way to mammals, although it must be noted the tissue locations of the octopamine uptake components were unknown.

The major inactivating enzymes in vertebrates are monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). The activities of both these enzymes have been shown to be low in the nervous tissue of locust (Vaughan and Neuhoff, 1976; Hayashi, Murdock and Florey, 1977), Drosophila (Dewhurst, Croker, Ikeda and McCaman, 1972), and honeybee brain (Evans and Fox, 1972). Although Richter and Rutschke (1977) have shown MAO activity in specific areas of the cockroach brain. In general, however, N-acetylation seems to be the major mechanism of inactivation, at least for DA, OA and 5-HT (Dewhurst et al., 1972; Vaughan and Neuhoff, 1976; Evans and Fox, 1972; Hayashi et al., 1977; Maranda and Hodgetts, 1977).

#### The Role of Cyclic Nucleotides

It is becoming apparent that several neurotransmitters and neuromodulators mediate some of their actions through



cyclic nucleotides. These act as intracellular second messengers, bringing about the appropriate cellular response by altering the activity of specific enzymes. Several criteria need to be fulfilled, however, before the involvement of the cyclic nucleotides can be concluded. This proof is not complete for any of the insect CNS responses, and in general amounts simply to the fact that biogenic amines can alter cyclic nucleotide levels in a nervous tissue homogenate.

Bodnaryk (1979, 1980) reported the presence of both a DA and OA-sensitive adenylate cyclase in the brain of the moth, Mamestra configurata. No evidence for these, however, was found in the nerve cord of the larva of the sphinx moth, Manduca sexta, where only 5-HT-sensitive adenylate cyclase activity was seen (Taylor, Dyer and Newburgh, 1976; Taylor and Newburgh, 1978, 1979). Taylor and Newburgh (1979) also found that several other putative neurotransmitters had no effect on cyclic AMP levels: NA, ACh, GABA, glutamate, glycine, glutamine, aspartate and adrenaline. Instead they found that several of these, namely: ACh, GABA, aspartate, glutamate, and glycine, all increased the level of cyclic GMP. OA, DA and 5-HT-sensitive adenylate cyclase activity has been located in the thoracic ganglia of the cockroach, Periplaneta americana (Nathanson and Greengard, 1973, 1974). NA stimulated activity as well, but only at much higher concentrations. By carrying out experiments in which they simultaneously added two of the amines, each at a concentration which maximally stimulated adenylate cyclase, Nathanson and Greengard (1973) were able to show that DA, OA



and 5-HT acted at different receptors, whereas the NA had no specific receptor and was simply exerting its effect through the receptors of the other amines. They also found that the  $\alpha$ -adrenergic blocker, phentolamine, was much more effective at blocking the increase in adenylate cyclase activity caused by DA, OA and NA than the  $\beta$ -adrenergic blocker, propranolol. The action of 5-HT on the cyclase was unaffected by both these antagonists, but was blocked by D-lysergic acid diethylamide (LSD), 2-bromo-D-lysergic acid diethylamide (BOL) and cyproheptadine. Conversely, these inhibitors were poor antagonists of the other amine responses. These workers also noted that the following substances were ineffective adenylate cyclase activators: tyramine, phenylethylamine, phenylethanolamine, carbachol, GABA, L-glutamate and histamine. Harmer and Horn (1977) studied adenylate cyclase in brain homogenates of the same cockroach (Periplaneta). They reported the presence of both DA and OA-sensitive cyclases but were unable to find any 5-HT-dependent activity. It is still unknown whether or not this difference does reflect a genuine difference in the distribution of the 5-HT receptors, or is simple due to different assay conditions. In contrast to all these results, Rojakovick and March (1972) reported only adrenaline and NA-sensitive adenylate cyclase activity in brain homogenates of another cockroach (Gromphadorhina portentosa).

Finally, it is worth remembering here that although some cockroach CNS synapses the biogenic amines may act via

cyclic AMP, at others calcium ions may be involved and at yet others, receptor activation may result in a decrease in cyclic AMP production. This situation has been well documented for vertebrate adrenoceptors and dopamine receptors (see Introduction of Chapter II, section II) and, for example, also occurs for the action of 5-HT in Aplysia (Pellmar, 1981). In fact, at the salivary gland of the blowfly, Berridge (1979; see also Introduction of Chapter II, section II) has proposed that 5-HT mediates its effect through two distinct receptors: one linked to calcium channels; and the other to the adenylate cyclase system.

#### Electrophysiological Evidence

Only a few studies have been carried out to determine the physiological action of biogenic amines on the insect CNS. Little is also known about the nature of the receptors since few potential agonists and antagonists have been tested.

In 1980 Usherwood et al found that OA depolarized freshly dissociated neurones from the thoracic ganglia of adult locusts. Roberts and Walker (1981), on the other hand, found that OA normally hyperpolarized neurones (DUM neurones) on the dorsal surface of the sixth abdominal ganglion of the cockroach. Sometimes, however, the hyperpolarization was followed by a depolarization which resulted in excitation. They also found that D(-)- and L(+)-OA were equipotent, whereas in most experiments the naturally occurring D(-) isomer is usually much more effective. Phentolamine reversibly blocked the response, as did cyproheptadine. This second antagonist in fact caused OA

to produce an excitation of the neurone. Perhaps more than one receptor type was involved in the OA response. Several more experiments are really required before anything can be concluded.

In the case of the catecholamines the first electrophysiological experiments were carried out by Twarog and Roeder (1957), using extracellular recording from the ventral nerve cord connectives and stimulation of the cercal nerve in the cockroach, Periplaneta americana. They found that low concentrations (0.1 mM) of adrenaline produced bursts of impulses and increased transmission at the cercal nerve - giant interneurone synapse. Higher concentrations (1 mM) greatly depressed this synaptic response. The responses to the equivalent concentrations of NA were similar except that the facilitation of the synaptic response at the low concentrations was not as marked. In 1963 Hodgson and Wright obtained similar results with adrenaline, NA and DA. By intracellular recording from the giant fibre axons they concluded that the blocking of activity at high concentrations was due the effect of the drugs at the cercal nerve - giant fibre synapse and that axon conduction was unaffected. This effect on synaptic transmission was not confirmed by Gahery and Boistel (1965) who found that DA only produced the bursts of impulses. Kerkut et al (1969b) recording intracellularly from cockroach sixth abdominal DUM neurones, bath applied DA, NA and adrenaline and reported that the cells were depolarized and excited.

Twarog and Roeder (1957) also applied 5-HT to their

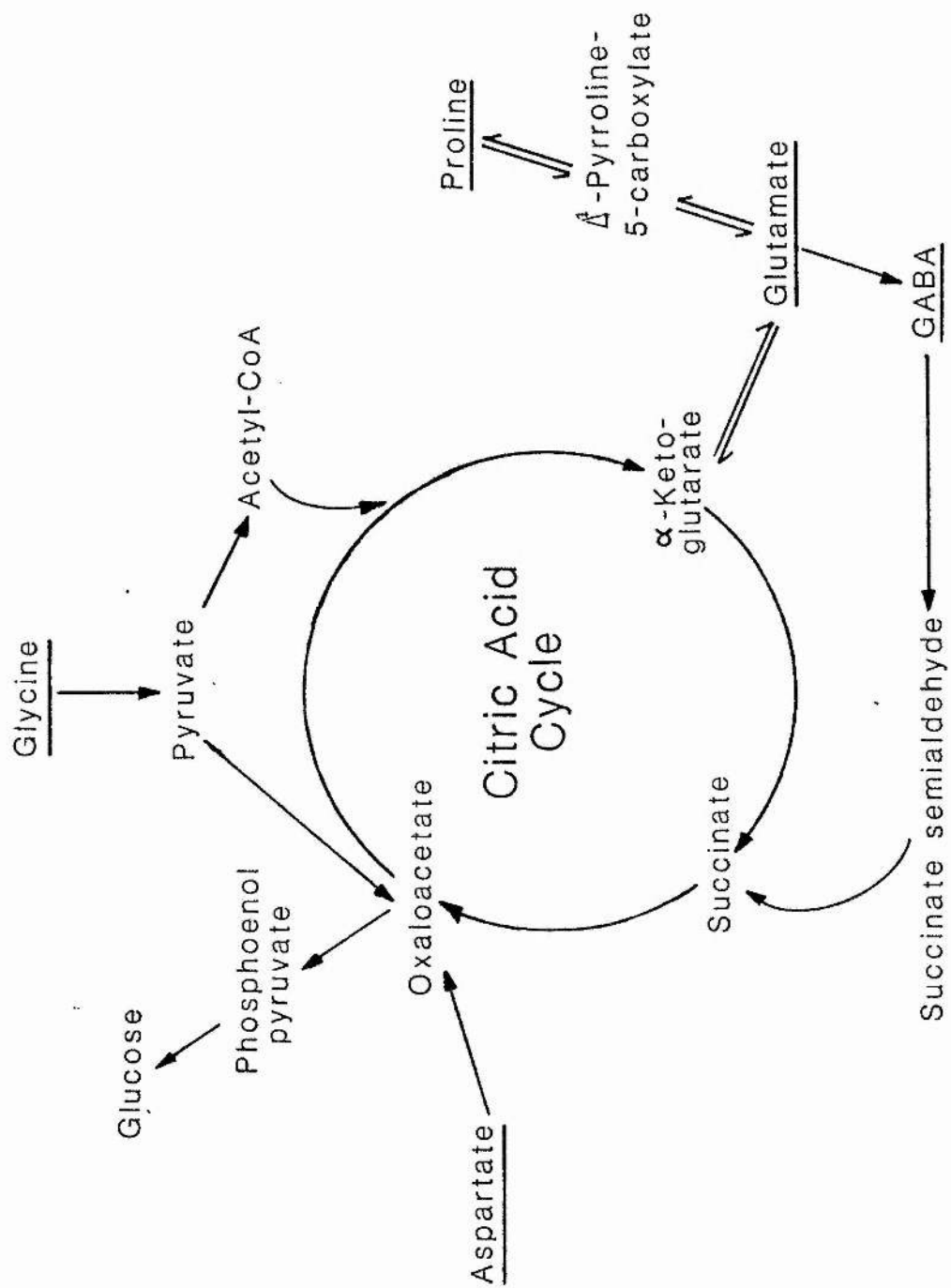
cockroach nerve cord preparation and observed facilitation of the giant fibre synaptic response. Hodgson and Wright (1963), however, found that 5-HT was completely inactive on the preparation. Using the same preparation but recording from the cercal motor nerve as well as the connectives Jégo, Jouan and Boistel (1970), reported that at low concentrations (10  $\mu\text{g/ml}$ ) 5-HT increased the efferent activity, whereas at high 5-HT concentrations (1  $\text{mg/ml}$ ) transmission was blocked. In 1970 Jégo and Boistel recorded intracellularly from the sixth abdominal DUM neurones. They found that prolonged exposure to 5-HT or tryptamine altered the action potentials they recorded in two respects: the after hyperpolarization was reduced; and an after depolarization developed. Along with these changes there was also an alteration in the firing pattern of the neurone in that the action potentials began to appear in bursts. They observed that calcium free saline also caused the after hyperpolarization to decrease and so hypothesized that the serotonin interfered with calcium and potassium ion movements during the action potential. In this respect it would be acting as a neuromodulator rather than a transmitter on these cells. This 5-HT response is similar to one of 5-HT's effects in the leech nervous system where it acts as a neurohormone, generating swimming by initiating a characteristic firing pattern in certain interneurones (Kristan and Nusbaum, 1983).

AMINO ACIDS

There are three amino acids that have been considered as possible transmitters in the nervous system : gamma-aminobutyric acid (GABA); glutamate; and aspartate. In the insect, GABA has by far received the most attention. Biochemical determinations have shown that it is present in significant amounts and also, it has been considered for several years to be the most likely candidate for the role of inhibitory transmitter at the insect neuromuscular junction (Usherwood and Grundfest, 1965).

Another reason contributing to this is that GABA is the only amino acid transmitter candidate that does not have a major role as a intermediary in energy metabolism, nor has a role as a building block in protein synthesis. Fig. 12 summarises the way in which some of the amino acids contribute to the production of energy. Glutamate, aspartate and glycine are readily converted into citric acid cycle intermediates. Using labelled glucose and/or glutamate a few groups of workers have found that labelled tricarboxylic acid intermediates were formed in substantial quantities in the nervous system (Bradford, Chain, Cory, and Rose, 1969; Treherne, 1960; Huggins, Rick and Kerkut, 1967), and in muscle (Huggins et al., 1967). It has been found in mammals that out of all the body tissues it is only the nervous system that contains significant quantities of GABA (Cooper, Bloom and Roth, 1978). Similarly, Osborne and Neuhoff (1974a) showed that in the cockroach, the concentration of GABA is about ten times less in muscle and blood compared with nervous tissue. And so it is thought that the

Figure 1.2 Diagram showing the involvement of some amino acids in cellular energy metabolism. GABA, glutamate and aspartate are major transmitter candidates. It may be seen that of these, only GABA does not have an important role as an intermediary in metabolism.





presence of GABA and its metabolic enzymes in specific parts of the nervous system indicate that it is acting as a neurotransmitter or neuromodulator in those areas.

Proline is another amino acid that has been shown to be present in fairly substantial quantities in a variety of insect nervous systems (Ray, 1964, 1965; Evans, 1975; Osborne and Neuhoﬀ, 1974a). However it is also found in high amounts in insect muscle and blood (Osborne and Neuhoﬀ, 1974a) and so is considered as a rich reserve energy source rather than a neurotransmitter candidate. It is through proline oxidation that the limitation of pyruvate at flight initiation may be relieved (Sacktor and Childress, 1967).

Of the other amino acids, glutamate is the only one that has been studied to any degree. It is thought to be the excitatory transmitter at the insect neuromuscular junction (Usherwood, 1978). Recently, however, it has been suggested that aspartate could function as the transmitter of the "slow" motoneurones at the neuromuscular junctions of the body wall muscles of fly (Musca domestica) larvae (Irving and Miller, 1980a, 1980b). As mentioned previously, motoneurones are known to make presynaptic connections in the CNS of insects. And so if Dale's hypothesis of synaptic unity is correct it may be expected that these amino acids also function as transmitters in the central, as well as the peripheral, nervous system.

Biochemical Evidence for the Transmitter Role of the Amino Acids

GABA is synthesized from glutamate using the enzyme glutamic acid decarboxylase, GAD. The level of GAD activity in the central nervous system has been studied in the following insects: honey bees (Frontali, 1961; Fox and Larsen, 1972); cockroaches (Baxter and Torralba, 1975; Huggins et al., 1967); locusts (Emson et al., 1974; Bradford et al., 1969); flies (Langcake and Clements, 1974) and sphinx moths (Maxwell, Tait and Hildebrand, 1978). Only Emson et al (1974) provided any evidence for the cellular localization of the enzyme. They found that in identified motoneurons of the locust, only the inhibitory ones showed the presence of GAD. This provides good evidence for the inhibitory transmitter role of GABA at the insect neuromuscular junction.

The cellular location of GABA itself has been studied in only two preparations: the cockroach brain (Frontali and Pierantoni, 1973) and the visual ganglia of the flies Musca and Drosophila (Campos-Ortega, 1974). In both cases autoradiography was used to locate the sites of tritiated GABA uptake. Frontali and Pierantoni (1973) found GABA in well defined cell groups, nerve fibre bundles, and neuropilar structures. They concluded that this resulted from uptake into GABA-specific cells. Campos-Ortega (1974), however, found GABA mainly accumulated in glial elements, and detected little in the neuronal elements. He also used tritiated glutamate and glycine. The uptake of glutamate was located in specifically in neuronal sites, whereas

glycine was distributed diffusely throughout the tissue. The reason for the different distributions of GABA uptake in the above preparations is unknown. Since the synaptic actions of amino acids are thought to be terminated by reuptake, however, perhaps the differing uptake distributions of GABA is indicative of different inactivating mechanisms in different brain areas.

Glutamate uptake at the insect neuromuscular junction has been studied by a number of groups (Faeder, Matthews and Salpeter, 1974; Botham, Beadle, Hart, Potter and Wilson, 1979; Faeder and Salpeter, 1970). They found that the rate of uptake was enhanced on stimulation of the muscle and that it was greatest at the glial sheath cells and the tracheoles in the junctional regions, as well as at the postsynaptic regions of the muscle. It was suggested that these results provided a possible mechanism for transmitter inactivation and a means of protecting the synapses from high blood glutamate. Osborne and Neuhoff (1974c) studied the accumulation of labelled proline, glycine, glutamate and GABA into cockroach metathoracic ganglia. They found that the rates were slow compared with certain vertebrate species, and that only the uptake of glutamate and GABA were significantly effected by temperature and metabolic inhibitors. Evans (1975), on the other hand, was able to distinguish between a sodium-sensitive and a sodium-insensitive component of glutamate uptake into the ventral nerve cord of the locust. He found that the sodium sensitive component showed saturation kinetics, was specific for L-dicarboxylic acids and was sensitive to some metabolic

inhibitors. He suggested that this system is located in the glial cells of the perineurium and associated with the insect blood brain barrier system.

### Electrophysiological Evidence

With regard to electrophysiological investigations, it is again GABA that has received most of the attention. In the early work, Suga and Katsuki (1961), recording externally from the anterior connectives of grasshopper prothoracic ganglia, found that the grasshopper's response to sound was inhibited by GABA and that this effect was antagonized by picrotoxin. In 1960 Vereshchagin et al showed that GABA caused an inhibition of the electrical activity of the ventral nerve cord of the pine moth caterpillar. Then in 1965, Gahery and Boistel found that transmission across the synapse between the cercal nerves and the giant interneurons was depressed by 10 mM GABA, whereas axonal conduction was unaffected. Later, in 1969, Steiner and Pieri, found that iontophoretically applied GABA also inhibited spontaneous electrical activity of ant brain neurones.

The above results suggest an inhibitory role for GABA but tells one little about the neurones or the cellular mechanisms involved. They also show that GABA, like ACh has a low potency when applied to the bathing medium. Again like ACh the two preparations that have been used to study the cellular actions of GABA are the DUM neurones, and the cercal nerve giant interneurone system of the cockroach sixth abdominal ganglion.

In 1969 (a and b), Kerkut et al showed that iontophoretically applied GABA hyperpolarized and depressed the spontaneous activity of the DUM neurones of the cockroach sixth abdominal ganglion. The size of the response was dose dependent, with the threshold being 10.5 pmoles. By altering the composition of the bathing saline they determined that the ionic basis of the GABA response was a substantial increase in the membrane's permeability to chloride ions and a slight increase in its permeability to potassium. In 1970, Pitman and Kerkut estimated, from the extrapolation of the graph of response amplitude versus membrane potential, that in normal saline the reversal potentials of the IPSP and of GABA were very similar:  $-78.9 \pm 2.1$  mV for the IPSP; and  $-75.4 \pm 0.6$  mV for GABA. They found that the two responses were reversed, both in chloride free saline and when the cells were impaled with microelectrodes containing 1M potassium chloride. Also, both reversal potentials were displaced towards the neuron's resting potential in a high potassium saline. They concluded from these results that the ionic mechanisms of the GABA response and the IPSP were similar. In addition, they also reported that both responses were blocked by the GABA antagonist, picrotoxin (100  $\mu$ g/ml). Takeuchi and Takeuchi (1969) have provided evidence, however, that picrotoxin does not in fact interact with the GABA receptor, but rather with the chloride ion channels, and so nothing can be deduced about the receptors involved in the responses. Walker, Crossman, Woodruff and Kerkut (1971), working on the same

preparation, found that the GABA antagonist, bicuculline (0.68 - 2.7  $\mu$ M), blocked both the IPSPs and the GABA response. They also found, however, that it also blocked the excitatory effect of the ACh agonist carbamylcholine. And so nothing can again be said about the receptors involved.

More recently, Roberts, Krosgarard-Larsen and Walker (1981) carried out a structure - activity study on the GABA receptors of the above preparation. They used conformationally restricted GABA agonists and determined their potency. They found that dihydromuscimol was the most effective agonist and concluded from the shape of this molecule that GABA interacts with its receptor in a partially extended form.

Goodman and Spitzer (1979, 1980) have studied the development of GABA chemosensitivity in the metathoracic DUM neurones of the grasshopper embryo. They found that neurones first become sensitive to GABA, either iontophoretically or bath applied, between day 8 and day 13 of the embryo's life, before in fact the neurones' own neurotransmitter is detectable. This response to GABA reversed at -70 mV, was abolished in chloride free saline, and was blocked by picrotoxin. These results, unlike those obtained with the embryonic ACh receptors, are very similar to those of adult cockroach DUM neurones. The reason for this difference is unknown.

Using the single fibre oil-gap technique and the cockroach sixth abdominal giant fibre preparation, Callec and Boistel



(1971a, 1971b) found that by stimulating cercal nerve ten they could evoke IPSPs in the giant fibres. Since IPSP amplitude depended on stimulus strength and the delay between the stimulus and the appearance of the IPSP was about twice that of a single chemical synapse, they decided that the IPSPs were caused by the stimulation of a group of inhibitory interneurons that connected the sensory cercal nerves to the giant fibres. GABA, when either iontophoresed or bath applied, caused membrane hyperpolarization, a reduction in EPSP amplitude, and a reduction in membrane input resistance of the giant fibres. The similarity of the GABA response and the IPSP, and the fact that they are both blocked by picrotoxin, has again led to the conclusion that the ionic mechanisms underlying both responses are similar. Hue, Pelhate and Chanelet (1979), have obtained evidence that GABA also acts on the presynaptic cercal afferents, causing a depolarization and a decrease in transmission to the giant fibres. They reported that this effect is dependent on the chloride and potassium ion concentrations and that it is blocked by picrotoxin. The underlying ionic mechanism is thus thought to be the same as the one for the GABA postsynaptic effect: a large increase in chloride conductance accompanied by a smaller increase in the potassium one.

By comparing the sensitivity of the cockroach giant interneurone synapses to the amino acids taurine and GABA under varying conditions, Hue, Pelhate and Callec (1981) have obtained indirect evidence for the existence of a specific GABA uptake mechanism. At this synapse, taurine



mimics the action of GABA. The decrease in the EPSPs and the increase in the membrane conductance due to GABA were enhanced by lowering the temperature of the perfusing saline, by using a sodium pump inhibitor (ouabain), and by using sodium-free salines. None of these conditions caused a significant change in taurine sensitivity. These results suggest the presence of an uptake mechanism which contains within it a sodium sensitive component. They also used two blockers of GABA uptake mechanisms, L-2,4-diaminobutyric acid (DABA) and  $\beta$ -aminobutyric acid (BABA). DABA specifically blocks neuronal GABA uptake in mammals, whereas BABA specifically blocks glial uptake of GABA. Only BABA produced any significant increase in GABA sensitivity and so they concluded that the uptake mechanism was present on the glial cells. This is probably one of the reasons for the low potency of bath applied as compared with iontophoretically applied GABA (10 mM and 10.5 pmoles, respectively).

Very little electrophysiological evidence exists for the central effects of the other amino acids. Usherwood et al (1980) found that glutamate hyperpolarized freshly dissociated locust thoracic ganglion neurones, whereas Steiner and Pieri (1969) found that glutamate usually caused excitation of neurones in the ant protocerebrum.

Kerkut et al (1969b) found that glycine hyperpolarized DUM neurones of the metathoracic ganglion of adult cockroaches. This was also the response observed by Usherwood et al (1980) with freshly dissociated neurones of adult locusts and cultured neurones from nymphal cockroaches.

The only report so far for the central effects of aspartate was that of Usherwood et al (1980), who found that the dissociated locust neurones could show either a hyperpolarizing or a depolarizing or a biphasic response.

In the last three sections the evidence for the neurotransmitter role of acetylcholine, the biogenic amines dopamine, noradrenaline, octopamine and serotonin, and the amino acids glutamate and GABA, in the insect nervous system has been reviewed.

There is more evidence to support the neurotransmitter role of acetylcholine in the insect central nervous system, than for any of the other candidates. It is thought to be released from the central projections of many sensory neurones and may also be used by some interneurones.

It is now generally accepted that the amino acids glutamate and GABA are, respectively, the excitatory and inhibitory neurotransmitters involved in neuromuscular transmission. It is also believed that GABA may act as an inhibitory central neurotransmitter. There is little evidence for the central role of glutamate, although information about this may come from studying the central projections of the excitatory motoneurones.

Dopamine, octopamine, noradrenaline and serotonin have all been detected within the nervous system of several insect species. In the periphery, it has been shown that octopamine is involved in the modulation of neuromuscular transmission and there is also reasonable evidence for the role of dopamine and serotonin in salivary gland activation. Little is known, however, about their physiological roles in the central nervous system.

The cockroach CNS probably contains dopaminergic neurones

since biochemical assays have shown dopamine to be present in significant quantities, but the localization of these neurones in the ventral nerve cord and suboesophageal ganglia is unknown. The ventral nerve cord and suboesophageal ganglia are less anatomically complex than the cerebral ganglia and may be better regions to focus on for intracellular recordings and dye injection.

One of the aims of this study, therefore, was to localize dopamine-containing neurones within wholemount ventral nerve cord and suboesophageal ganglia of the cockroach. This was achieved using a fluorescence-based technique which is able to reveal dopamine, noradrenaline and serotonin. The colour of the fluorescence indicated that no serotonin-containing neurones were detected. Dopamine and noradrenaline could not be distinguished, although other evidence suggests that most, if not all, of the neurones are dopaminergic.

Several attempts were made to further characterize a few of the neurones by combining dye infusion or intracellular dye injection with the fluorescence technique. Although some success was achieved, especially with fluorescence-dye infusion combination, difficulties were encountered with the identification and filling of the neurones.

Electrophysiological experiments showed that an identified prothoracic neurone was depolarized in response to pressure ejected and bath applied dopamine and noradrenaline. Some evidence is provided that catecholamine receptors are present on the neurone but the possibility that dopamine could be acting at octopamine or acetylcholine receptors

cannot be ruled out. Data is also presented which suggests that, in this case, dopamine does not act through the second messenger, cyclic AMP.

Additional experiments to determine the effect of pressure ejected glutamate and GABA on the membrane potential of an identified metathoracic motoneurone were also carried out. The effects of picrotoxin and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid on the amino acid responses and the membrane properties of the neurone somata were investigated.

A preliminary account of some of the above work has previously been published (Fleming and Pitman, 1983).

CHAPTER 1

THE LOCALIZATION AND IDENTIFICATION OF BIOGENIC AMINE  
CONTAINING NEURONES IN THE COCKROACH VENTRAL NERVE CORD.

SECTION I

DISTRIBUTION OF BIOGENIC AMINE-CONTAINING NEURONES IN THE  
COCKROACH VENTRAL NERVE CORD AS DEMONSTRATED BY THE  
GLYOXYLIC ACID TECHNIQUE.



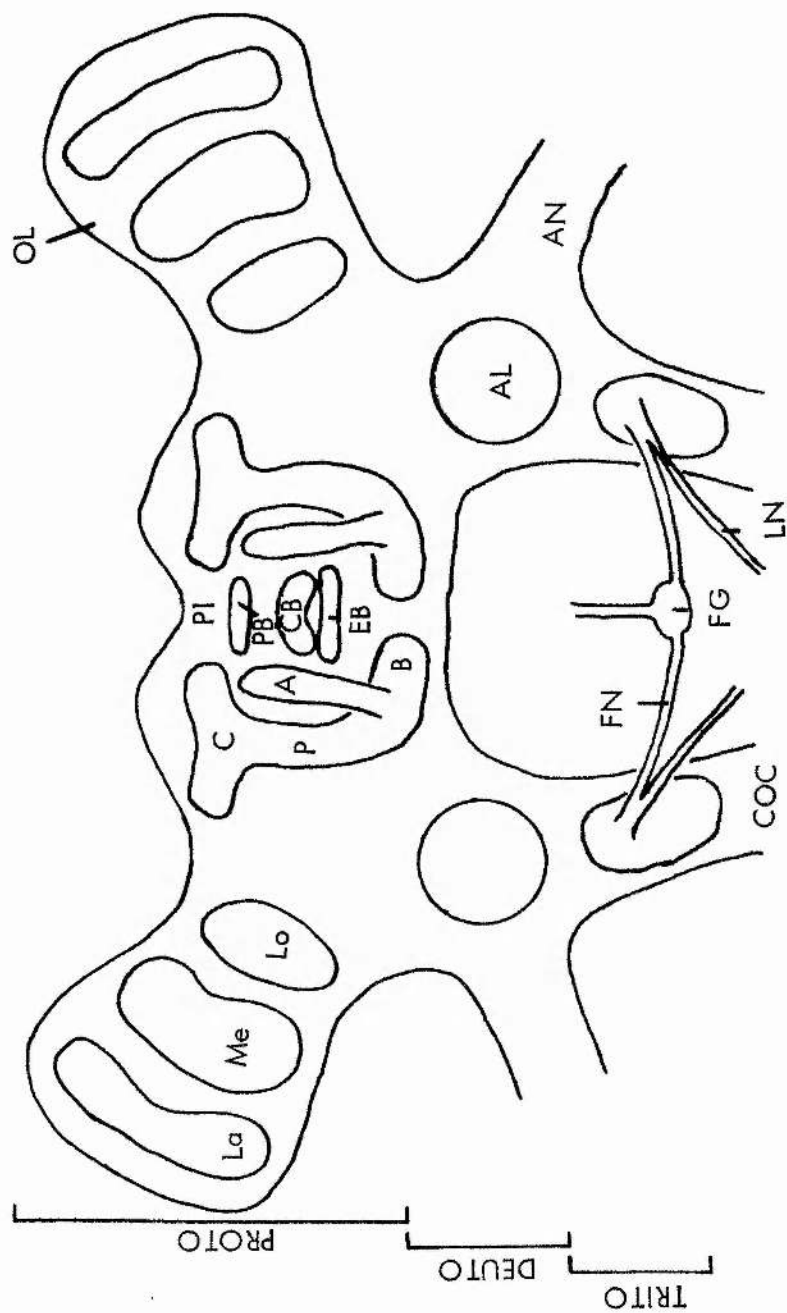
## INTRODUCTION

A new era in monoamine research began with the introduction of the formaldehyde induced histochemical fluorescence method of Falck-Hillarp (Falck, 1962; Falck, Hillarp, Thieme and Torp, 1962). This method allowed the visualization of central monoamines for the first time and provided estimates of terminal and cell body density distribution in mammalian central nervous tissue (Dahlström and Fuxe, 1964a, b). Several groups of workers using this method have demonstrated fluorescent neurones and fibre bundles in the brains of various insect species.

Briefly, the insect brain can be divided into three main regions (Fig. 1.1): the protocerebrum, which is the largest and includes the optic ganglia; the deutocerebrum, which gives rise to the roots of the antennal nerves; and the tritocerebrum, the smallest region, which connects the brain to the stomodeal nervous system. Catecholamine-containing neurones have been found in all regions of the brain's cell body layer in every insect species studied so far (Klemm, 1976). Catecholamine-containing fibres have been located in the 'unstructured' neuropile (which consists of loosely arranged nerve fibres) of locusts, grasshoppers, moths, flies, bees and cockroaches (Klemm, 1976). 'Glomerular' (or 'structured') neuropile consists of readily identifiable structures which contain high concentrations of nerve terminals. With the <sup>advent of</sup> the Falck-Hillarp technique catecholamine-containing fibres have been shown to be present in several of these structures, including the optic lobe, the central body complex and the mushroom bodies. The precise

Figure 1.1 Diagram of an insect brain illustrating its general layout and the positions of the 'structured' neuropile regions. The various regions of the corpus pedunculatum (mushroom bodies) are as follows: A,  $\alpha$ -lobe; B,  $\beta$ -lobe; C, calyx; P, pedunculus. The regions of the optic lobe are: OL, optic lobe; La, lamina; Me, medulla; Lo, lobula. Other labelling: PROTO, protocerebrum; DEUTO, deutocerebrum; TRITO, tritocerebrum; PI, pars intercerebralis; PB, protocerebral bridge; CB, central body; EB, ellipsoid body; AL, antennal lobe; FG, frontal ganglion; NC, frontal connective; NL, labral nerve; COC, circumoesophageal connective, AN, antennal nerve.

(Adapted from Klemm, 1976)



distribution of the fibres in the glomerular structures varies in different insect species (Elofsson and Klemm, 1972; Frontali, 1968; Frontali and Norberg, 1966; Klemm and Björklund, 1971; Klemm, 1976; Klemm, 1983).

The Falck-Hillarp technique requires the tissue to be freeze dried, reacted with formaldehyde vapour, embedded in paraffin and sectioned. A relative humidity of 50-60% is essential for optimum results (Falck and Owman, 1965). The biggest drawbacks with this method are that it takes several days to process the tissue and it is very difficult to obtain consistently good fluorescent preparations.

Ten years after introduction of the Falck-Hillarp technique another very productive step forward was initiated with the introduction of a much quicker and simpler fluorescence technique: the Vibratome glyoxylic acid method (Axelsson, Björklund, Falck, Lindvall and Svensson, 1973; Lindvall, Björklund and Svensson, 1974; Lindvall and Björklund, 1974; Lindvall, Björklund, Hökfelt and Ljungdahl, 1973). A vibratome can be used to obtain 20-35  $\mu$ m thick sections of fixed or fresh tissue. There is no need to freeze or embed the tissue beforehand; it is simply glued to the holder of the vibratome, immersed in an ice-cold buffer solution, and then cut by the machine at a preset vibration rate. For the fluorescence technique the next step is to incubate the sections in an ice-cold glyoxylic acid (GA) solution. They are then dried and finally heated (alone or in the presence of GA vapour) to bring about the reaction between the GA and the endogenous monoamines. This technique reduced the

processing time from several days to several hours. Also, although the reactions involved and the fluorophores formed are similar to those of the Falck-Hillarp method, the GA technique was found to be considerably more sensitive (5-10 times), and the fluorescence itself longer lasting (Lindvall and Björklund 1974)

The next step forward occurred in 1976 with the introduction of a modified GA condensation reaction (de la Torre and Surgeon 1976a and b). Cryostat sections of tissue were exposed to a room temperature solution containing sucrose, potassium phosphate and glyoxylic acid. The specimens were then air dried and heated in an oven. By this method fluorescent brain sections were produced in less than 30 min.

More recently the GA technique was further modified for the staining of unsectioned leech ganglia (Stuart, 1981; Lent, 1982). Using this procedure the connective tissue is cleared and there is excellent visualization of the serotonergic and dopaminergic cell bodies, axons and endings.

MATERIALS AND METHODSTHE GLYOXYLIC ACID TECHNIQUE

Product formation for all the fluorophore-forming compounds follows a similar reaction scheme which proceeds in two steps (Björklund, Lindvall and Svensson, 1972; Lindvall et al., 1974). The reaction scheme for the primary catecholamine dopamine (DA) is illustrated in Fig. 1.2. The steps are as follows:

1. A Pictet- Spengler type cyclization between DA and one GA molecule to form the weakly fluorescent 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid, via a Schiff's base. (1-4)

2. Reaction of this compound with a second GA molecule, in an intramolecular acid catalysed reaction, to yield the strongly fluorescent 2-carboxymethyl-6,7-dihydroxy-3,4-dihydroisoquinolium compound. This is in a pH-dependent equilibrium with its tautomeric quinoidal form. (4-8)

Product formation for other fluorophore-forming compounds follows a similar reaction scheme.

With this technique dopamine and noradrenaline (NA) (Fig. 1.3a) give a blue to green fluorescence and serotonin (5-HT) (Fig. 1.3b) a yellow to orange fluorescence depending on the filter system used in the microscope. Secondary catecholamines, such as adrenaline (Ad) (Fig. 1.3c), give an insignificant fluorescence yield in the GA reaction. The tetrahydroisoquinoline derivatives formed in the first stage of the scheme cannot react with a second GA molecule

Figure 1.2 Fluorophore formation from dopamine by glyoxylic acid. GA, glyoxylic acid; DA, dopamine. For explanation see text.



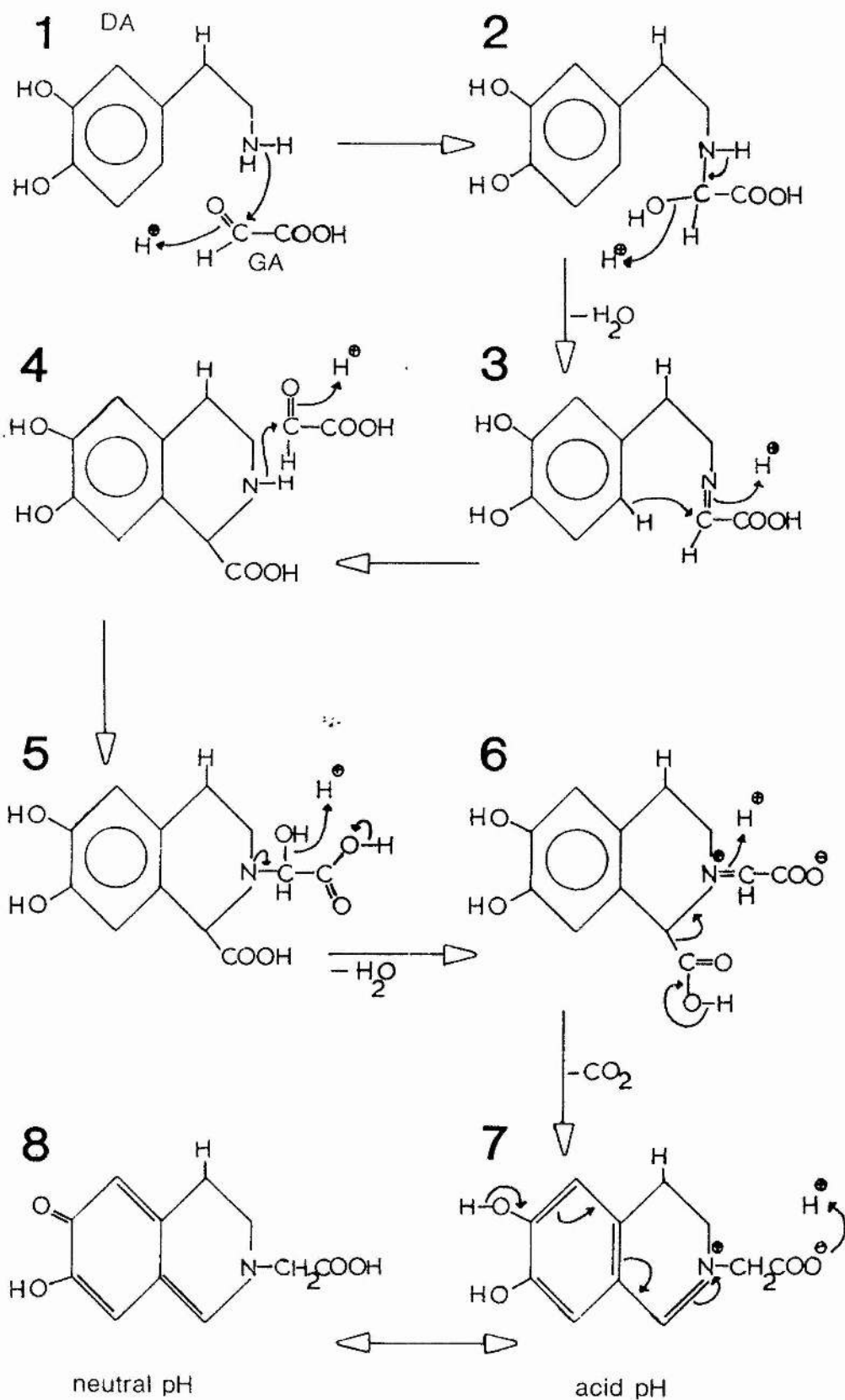
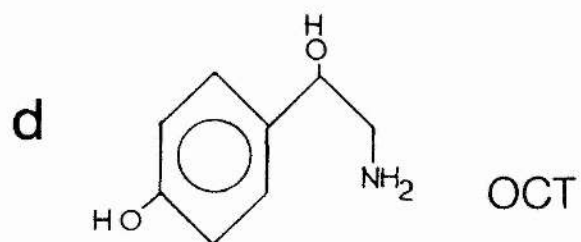
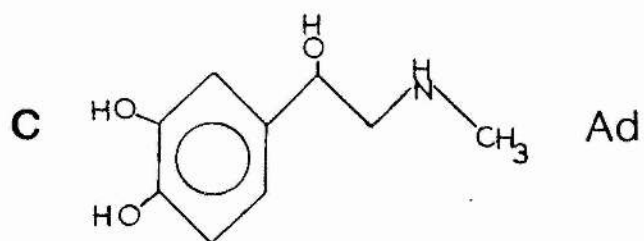
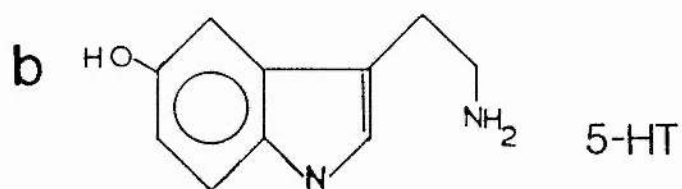
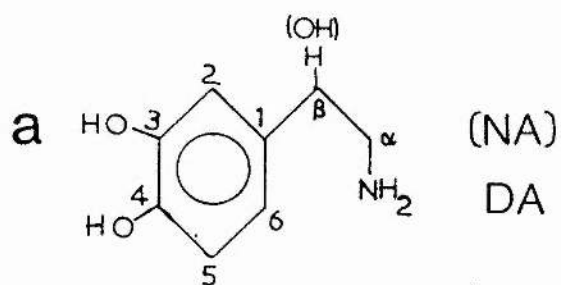


Figure 1.3 Structures of some biogenic amines. Labelling used for the positions in phenylethylamines is also shown.



due to the presence of the N-methyl group, and so the highly fluorescent second stage derivatives are not formed. Phenolamines, such as octopamine (OA) (Fig. 1.3d), also cannot be demonstrated. The cyclization is favoured by substituents on the aromatic ring giving a high electron density at the point of ring closure (the 6-carbon). This is fulfilled in phenylethylamines having a hydroxy group para to the position of closure, that is in the three position. OA is unsubstituted at this point and hence is unreactive, whereas both DA and NA give high yields of the reaction products.

#### DEVELOPMENT OF THE GA TECHNIQUE FOR USE WITH WHOLE MOUNT COCKROACH NERVE CORD.

As mentioned in the introduction, the GA technique was first introduced by Björklund and his co-workers in the early 1970's for use with brain slices. It was then modified by Stuart (1981) for whole mount leech nerve cord. Hence it was decided to carry out a few preliminary experiments on the leech for comparative purposes before developing the technique further for use with the cockroach. Leech ganglia contain both DA and 5-HT cells, so a comparison of the colour of the fluorescent cells in each animal provided a way of determining whether the cockroaches' fluorescent neurones contained catecholamine or 5-HT.

#### The Preparation

Leeches (species unidentified) were collected from the local burn and stored in the refrigerator at 4°C until required.

The dissection was as follows: The animal was pinned down on a Sylgard resin block dorsal side uppermost. The skin was slit down the midline using a scalpel and pinned out on each side. The gut was then pulled out to expose the underlying nerve cord which was kept moist with saline. Some of the ventral ganglia, with the associated lateral nerves, were excised using small dissection scissors and finely ground watchmakers forceps and placed in some saline. Finally, the ventral sinus which surrounds the nervous cord was carefully removed.

#### Saline

The composition is given in Table 1.1

#### GA Solution

The composition used for this set of experiments is given in Table 1.2a.

It was always prepared fresh just prior to the start of experimentation.

In all cases the reagents were dissolved in distilled water, titrated with 3M sodium hydroxide to a pH of 7.4, and then made up to a final volume of 10ml with more distilled water.

#### The GA Procedure for the Leech Specimens

The protocol for this set of experiments is given in Table 1.3.

Each experiment was carried out on a glass backed aluminium slide. These were made from rectangular pieces of thin aluminium from which a central hole had been cut out. One of the glass covers used for microscope slides was glued

TABLE 1.1

SALINE COMPOSITION

Sodium Chloride	214mM
Potassium Chloride	3.1mM
Calcium Chloride	9.0mM
N-tris-(Hydroxy-methyl)methyl-2-aminoethane Sulphonic Acid (TES)	10mM

TABLE 1.2a

GA SOLUTION COMPOSITION FOR THE LEECH EXPERIMENTS

Sucrose Crystals	0.68g (0.20M)
Potassium Phosphate Monobasic ( $\text{KH}_2\text{PO}_4$ )	0.32g (0.24M)
GA Monohydrate	0.10g (1%)

TABLE 1.2b

GA SOLUTION COMPOSITION FOR THE COCKROACH EXPERIMENTS

Sucrose Crystals	0.32g (0.10M)
$\text{KH}_2\text{PO}_4$	0.16g (0.12M)
GA Monohydrate	0.40g (4%)



TABLE 1.3

GA PROCEDURE FOR LEECH PREPARATIONS

Incubate ganglia in the GA solution for 1.5 mins.

Suck off as much solution as possible using a syringe.

Blow dry (cool) for 30 min.

Cover tissue with a couple of drops of mineral oil.

Incubate in a 95° oven for 5 min.

Wash off the oil using xylene.

Mount in DPX.

to one side of the aluminium. This could easily be removed by soaking the slide in acetone and thus the slide could be reused.

The specimens were observed using a Leitz Dialux 20 microscope set up for incident light fluorescent microscopy. It was equipped with a mercury vapour lamp and a Leitz filter block D, which contains a BP 355-425 exciting filter and a LP 460 suppression filter. The specimens were also observed under normal transmitted light.

Photographs were taken with a Wild 45/51S Photoautomat equipped with a 35mm magazine on either Kodak Ektachrome 200 ASA film or Ilford FP4 125 ASA film. The details for the development of the Ilford film are given in Table 1.4.

Examples of the fluorescence are shown in Figs. 1.4a and 1.4b.

Fig. 1.4a shows six yellow fluorescing cells. The large midline pair (called Retzius cells) are multimodal effectors; the small lateral pairs are interneurons. All of these cells are known to contain 5-HT (Rude, Coggeshall and van Orden, 1969; Lent, Ono, Keyser and Karten, 1979). Fig. 1.4b shows a pair of cells. The blue-green cell, seen along with part of its peripheral axon projection, is thought to contain DA (Stuart, Hudspeth and Hall, 1974; Lent 1982). The yellow cell is a small lateral interneurone.

These results agreed with those obtained by previous workers using either the Falck-Hillarp or the GA techniques. And so the next stage was to develop the GA technique further, for

TABLE 1.4

DETAILS OF DEVELOPING PROCEDURES

FILM	DEVELOPER (DILUTION)	TIME FOR DEVELOPING	FIXER	TIME FOR FIXING
KODAK AHU MICROFILM 5460	KODAK D-76 (1:2)	12 MIN.	ILFORD HYPAM RAPID FIXER (1:4)	2 MIN.
ILFORD FP4	KODAK D-76 (1:1)	8 MIN.	ILFORD HYPAM RAPID FIXER (1:4)	2 MIN.
KODAK ORTHO FILM TYPE 3	ILFORD PQ UNIVERSAL (1:9)	1.5-3 MIN.	ILFORD HYPAM RAPID FIXER (1:4)	2 MIN.

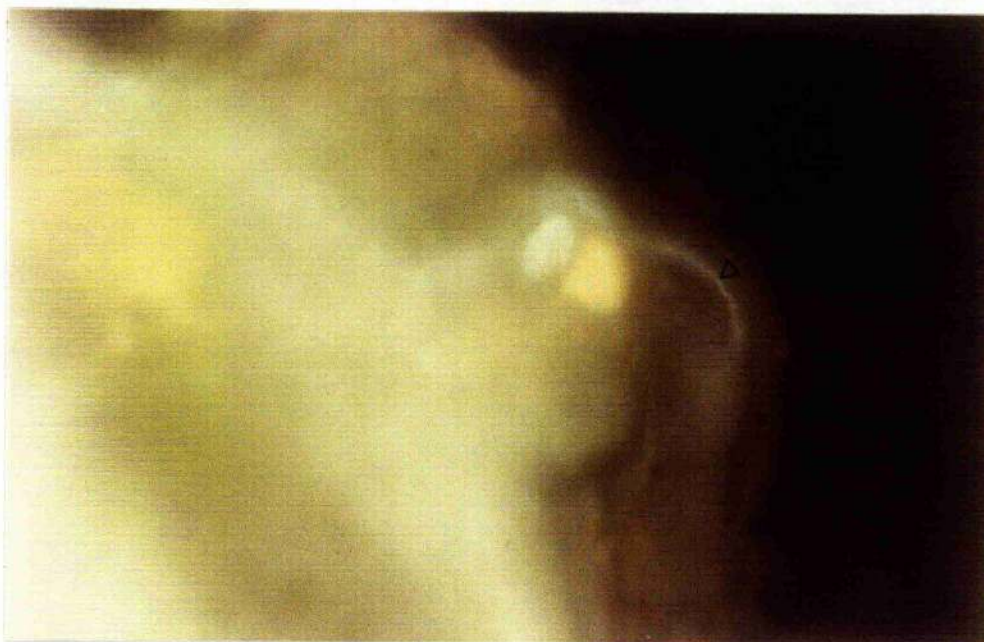
Figure 1.4, a and b Fluorescence of a midbody leech ganglion after being treated with glyoxylic acid.

(a) Whole ganglion. Six yellow (serotonergic) fluorescing nerve cell bodies are clearly seen. Calibration 100  $\mu$ m.

(b) Right lateral portion of ganglion. At the edge of the ganglion there is one yellow (serotonergic) neurone and one blue-green (dopaminergic) neurone with part of its peripherally projecting axon ( $\triangleright$ ). Calibration 25  $\mu$ m



**a**



**b**

use with whole mount cockroach nerve cord.

### The Preparation

Cockroaches (Periplanata americana) were maintained under warm conditions and reared on a diet of Minced Morsels dog food and water. All experiments were done either on adult males or on young nymphal cockroaches (4-7mm in length) of both sexes.

The dissections were as follows:

(i) Adult cockroaches - thoracic and abdominal ganglia. The head and legs were removed and the animal pinned down on a Sylgard block ventral side uppermost. Saline was used throughout to keep the preparation moist. The abdominal ganglia were exposed by cutting down the abdominal sterna on both sides of the midline, gripping the last sternite with forceps and lifting them all free by carefully separating them from the underlying connective tissue. The sterna were then cut loose. Each of the thoracic ganglia was exposed by first cutting the furcasternite and then cutting down both sides of the basisternite. This allowed the sternum to be lifted free. Some of the fat, connective tissue and tracheae which cover the nerve cord were cleared away. The cord was excised by cutting the lateral nerves on both sides of all the ganglia, gripping the abdominal end with forceps, and gently pulling it free from the rest of the animal. It was then placed in some saline and the rest of the tracheae and adhering connective tissue were carefully removed. The next step was to remove the neural lamella from each of the thoracic ganglia in turn. To do

this, the nerve cord, ventral side up, was placed on a piece of black plastic with the ganglion to be desheathed lying across an Araldite ridge. The posterior and anterior connectives of the ganglion were held down by elastic bands. A drop of 20% methylene blue was applied to the surface and then washed off. The sheath, which was now visible, was grasped close to the midline with two pairs of watchmakers forceps and torn by slowly pulling the forceps apart. This was continued until most of the ventral surface was desheathed. This whole process was then repeated for the remaining thoracic ganglia.

(ii) Nymphal cockroaches - thoracic and abdominal ganglia. The dissection procedure was very similar to that of the adults except that the nymphal<sup>cockroaches</sup> were anaesthetized with CO<sub>2</sub> and no desheathing was carried out. It was, of course, a much more delicate operation with fine forceps and dissection scissors being used throughout.

(iii) Adult cockroaches - suboesophageal ganglion. The animals were anaesthetized with CO<sub>2</sub>. The legs were removed, the antennae cut off and the animal pinned out, ventral side uppermost and head and neck stretched out, on a Sylgard block. The furcasternum of the prothoracic ganglion was cut in two. The posterior edge of the bastisternum was grasped by forceps and slowly lifted off as the sides were being snipped up. This cutting was continued up the ventral sclerites of the neck and onwards until the labrum was also lifted clear. The maxilla were snipped off and the surrounding tracheae and salivary duct carefully



removed. Both sides of the ventral surface of the head capsule were cut from their posterior edge to the start of the mandibles. The anterior connectives of the prothoracic ganglion were cut near their exit point, grasped with forceps and lifted up to expose the bridge of cuticle which connects the top and bottom surfaces of the head capsule. This was cut and the ventral surface of the head capsule lifted clear. The anterior connectives were held again and slowly pulled up. This allowed the suboesophageal connectives to be cut near to the supraoesophageal ganglion and then all the peripheral nerves to be cut as well. The ganglion was placed in some saline, the adhering connective tissue and tracheae removed and the ventral surface desheathed as described previously.

#### GA Solution

The composition used for the cockroach experiments is given in Table 1.2b.

It was prepared as described previously.

#### The GA Procedure

For most experiments, the ganglia were pretreated before incubation in the GA solution. In the case of the nymphs this involved a 5 min incubation in distilled water or a 5 or 15 min incubation in calcium-free saline containing 4mM ethyleneglycol-bis( $\beta$ -aminoethyl ether)N,N'-tetraacetic acid (EGTA). The adult ganglia were incubated in this solution for 30 min. For most of the experiments with the suboesophageal ganglia the monoamine oxidase inhibitor nialamide, (1mg/ml), was also added to the pretreatment

saline. The pretreatment was carried out on the same aluminium slide as the GA incubation.

The protocols for the nymph and adult experiments are given in Tables 1.5 and 1.6 respectively.

Control experiments were run in which the GA was omitted from the GA solution.

For some experiments the cockroach nymphs pretreated with an injection of either nialamide (Sigma) and L- $\beta$ -3,4-dihydroxyphenylalanine (L-DOPA; Sigma), colchicine (Sigma) or reserpine (Sigma) as follows.

(i) Nialamide and L-DOPA.

5mg of nialamide and 10mg of L-DOPA were dissolved in a final solution containing 1.5ml saline and 30ul 1M hydrochloric acid (HCl). The HCl was required to dissolve the nialamide. Injections were carried out 2-3 days prior to experimentation.

(ii) Colchicine.

0.5% colchicine in saline was injected 3-4 days prior to experimentation.

(iii) Reserpine.

4mg of reserpine was dissolved in a final solution containing 600 ul of 1M ascorbic acid, 600 ul distilled water and 10ul 3M sodium hydroxide. The ascorbic acid was needed to dissolve the reserpine, the solution then being neutralised with sodium hydroxide. Injections were carried out 3-4 days prior to the start of experimentation. In this case control animals, injected with the same

TABLE 1.5

GA PROCEDURE FOR NYMPHAL COCKROACH PREPARATIONS

Pretreat ganglia as described in text.

Suck off the solution using a syringe.

5 min incubation in the GA solution.

Suck off the solution.

Blow dry (cool) for 50 min.

Cover the tissue with mineral oil.

Incubate in a 90<sup>o</sup> oven for 5min.

Dissolve off the oil with xylene.

Mount in DPX.

TABLE 1.6

GA PROCEDURE FOR ADULT COCKROACH PREPARATIONS

Pretreat ganglia as described in text.

Suck off as much solution as possible using a syringe.

GA solution injected into the neuropile using a syringe equipped with a broken tipped microelectrode. 30 min incubation in this solution.

Suck off the solution.

Blow dry (cool) 1.25 hrs.

Cover tissue with mineral oil.

Incubate in a 90° oven for 5 min.

Dissolve off the oil with xylene.

Mount in DPX or xylene.

solution but minus the reserpine, were also set up.

In each case, a syringe equipped with a broken tipped microelectrode was used to inject the appropriate solution into the abdominal region of the nymphs.

The specimens were observed and photographs taken as described previously for leech ganglia.

RESULTSTHORACIC AND ABDOMINAL GANGLIA

After treatment with GA it was found that each ventral ganglion of the cockroach contained a relatively small number of bilaterally symmetrical fluorescent neurone somata (Fig. 1.5). It may be seen that the fluorescent cells of the abdominal ganglia show serial homology (Fig. 1.5). This is not the case with the thoracic ganglia, where for example, the prothoracic ganglion contains four pairs not seen in the other two. The fluorescence photomicrographs depicted in Fig. 1.6 show typical prothoracic ganglion preparations.

The neuropile region of each ganglion contains an extensive network of varicose fibres (Figs. 1.7a, b, 1.8, 1.9, 1.10, 1.11 and 1.12). This is especially clear in the abdominal ganglia (Figs. 1.7a, b, 1.8, 1.9 and 1.10) where the tissue is thinner and results in the specific fluorescence being less masked by the general background tissue autofluorescence. In the case of the thoracic ganglia the fibres are clearest around the edges of the ganglia (Fig. 1.11 and 1.12), where the tissue is at its thinnest.

Fluorescent axons are visible in the connectives joining all the adjacent ganglia (Figs. 1.7a, b, 1.8, 1.9, 1.10 and 1.11). Some of these axons branch once they enter a ganglion and soon become lost in the complex fibre network and the background fluorescence of the neuropile (Fig. 1.10); others can be seen to pass right through (see for example Fig. 1.7a, b and 1.13). Again this was most obvious in the thinner abdominal preparations. In general a greater number

of fluorescent axons were seen to enter the thoracic ganglia (about 10) compared with the abdominal ones (about 6). Figs. 1.14 and 1.15 especially, show identically positioned axons in the pair of connectives, some of which presumably originate from more anterior bilaterally symmetrical cells.

Each cell is one of a bilaterally symmetrical pair and these will presumably have an equivalent role in the opposite halves of the ganglion. The same can be said for the pairs of cells showing serial homology. In the thoracic ganglia the neurone pairs are not all identical. The unique pairs could be involved in the processing of information used by, or sent out from, that one particular ganglion; or they could send processes out into the other ganglia and by that way have a similar function in each (cf. the interneurons involved in locust flight, Robertson and Pearson, 1984).

In general the axons of the fluorescent cells could not be followed. The only exception was the fairly large pair of fluorescent medial cells of the prothoracic ganglion where the axon could be seen emerging from the cells. Focusing up and down at high power on the microscope indicated that the axons headed into the posterior connectives, but this was not certain. These cells were also the largest, being about 20  $\mu\text{m}$ , and among the best candidates for intracellular studies in the thoracic and abdominal nerve cord.

With one exception, no fluorescent fibres were seen in the peripheral nerves. The exception was found in the sixth abdominal ganglion where in two preparations a single



abdominal ganglion where in two preparations a single varicose fibre was seen to run into each cercal nerve (Fig.1.16).

In experiments in which the nymphal animals had been pretreated with an injection of nialamide and DOPA or colchicine, there was only a very slight increase in specific fluorescence.

In the case where the nymphal animals had been pretreated with an injection of reserpine there was a substantial decrease in the specific fluorescence (Fig. 1.17).

#### SUBOESOPHAGEAL GANGLIA

Figs. 1.18 and 1.19 show that this ganglion contains 23 fluorescent neurones. Most of the cells are bilaterally symmetrical and in general very small; less than 15 $\mu$ m. There is one unpaired cell which is located on the midline, anterior to the centre point. By carrying out experiments in which the ganglia were halved horizontally before the GA incubation, it was found that this was also the only fluorescent cell to be found on the dorsal surface. Fig. 1.20 shows an enlarged view of the anterior ventral surface of the ganglion. The large anterior pair are about 30  $\mu$ m; the unpaired neurone (seen more clearly from the dorsal surface) about 20  $\mu$ m. Bilaterally symmetrical varicose fibres, which originate from the cerebral ganglion and the large pair of fluorescent neurones, are also seen in the two halves of the ganglion. Fig. 1.21 shows the dorsal unpaired neurone and some fluorescent varicose fibres at a higher

magnification. Part of the cell's axon is also seen. It is worth noting here that Klemm(1971) located some dopamine-containing neurones on the dorsal surface of the thoracic and abdominal ganglia of Trochoptera. They are paired and lie medially, although their positions are variable. Each neurone gives rise to a single neurite which then bifurcates and extends into the neuropile on both sides of the ganglion. It is not known if the branches extend into any peripheral nerves.

It appears from Figs 1.20 and 1.21 that there are far fewer fluorescent fibres in the neuropile of this ganglion than in the thoracic ganglia described before. All these experiments, however, were carried out on the suboesophageal ganglion (SOG) of adult animals, whereas the previous pictures showing the numerous fibres were all from young nymphs. Nymph SOG are very small and fragile and extremely difficult to dissect out. Adult ganglia, apart from being a lot thicker, were subject to a harsher pre-GA incubation treatment. This results in the preparations where only a few of the total number of fibres are preserved and clearly seen. Fig. 1.22 shows the dorsal unpaired neurone and part of its axon. It is clearly seen to bifurcate soon after it emerges from the cell body. Focusing up and down at high power indicated that each branch of the axon went into the ipsilateral circumoesophageal connective, but also as before, it was not absolutely clear.

The SOG was the only ganglion which consistently showed peripheral nerves containing fluorescent axons. One pair of

posteriorly located nerves, each containing two fluorescent axons were seen. Fig 1.23 shows one of the axons is strongly fluorescent, the other much weaker. It was concluded that these are the nerves which innervate the salivary gland since it is already known that these nerves do in fact emerge from the dorso-lateral edges of the ganglion (Whitehead, 1971) and that all the evidence so far indicates that the transmitter is dopamine (see for example Bland, House, Ginsborg and Laszlo, 1973; House and Smith, 1978). Although it was impossible to tell in these experiments from which fluorescent cells the fibres originated (since once they entered the ganglion the fluorescent axons became lost in the neuropile), they were later identified using retrograde staining with hexaminecobaltic chloride (see Section IV).

The only fluorescent axons that could be seen clearly for any distance were those of the larger anterior lateral pair. Fig. 1.24 shows the cells and their axons. These run into the posterior connectives.

Several fluorescent axons were seen in the circumoesophageal connectives. These connectives are joined by two commissures, and it is through these that nerve fibres that emerge from the glomeruli of both sides of the tritocerebrum (the most posterior section of the cerebral ganglia) almost entirely cross over to the other side (Willey, 1961). Slightly more anterior to the first commissure, two nerves, the frontal connective and the labral nerve, emerge from a common root on the two sides of

the tritocerebrum (Willey, 1961). Both the commissures and the nerves are shown in Fig. 1.25. Fluorescent axons were seen in the smaller anterior commissure and in the frontal connectives (Fig. 1.26). Fluorescent fibres were seen to run through the ganglion, between the circumoesophageal connectives, but it impossible to tell if these fibres were present in the first commissure since this structure can not be visibly separated from the main body of the ganglion after GA treatment. Klemm (1983) also observed fluorescent fibres in the frontal connectives; on reaching the frontal ganglion the fibres aggregate at the periphery of the neuropile.

All the neurones and the fibres that have been described above showed the same colour fluorescence, blue-green. This was similar to that of the dopamine-containing neurones of the leech which were described previously. It is likely, therefore, that these cells contain a catecholamine. In the thoracic and abdominal ganglia no neurones were seen with the yellow-orange fluorescence characteristic of 5-HT. In the SOG, however, a few small, rapidly fading yellow-orange fluorescent neurones were seen. These were in similar positions to some of the 5-HT neurones recently located by Bishop and O'Shea (1983) using immunocytochemistry (Fig. 1.27).

Figure 1.5 Ventral view of the cockroach ventral nerve cord showing position of catecholamine-containing nerve cell bodies. Th, thoracic ganglia; Ab, abdominal ganglia.

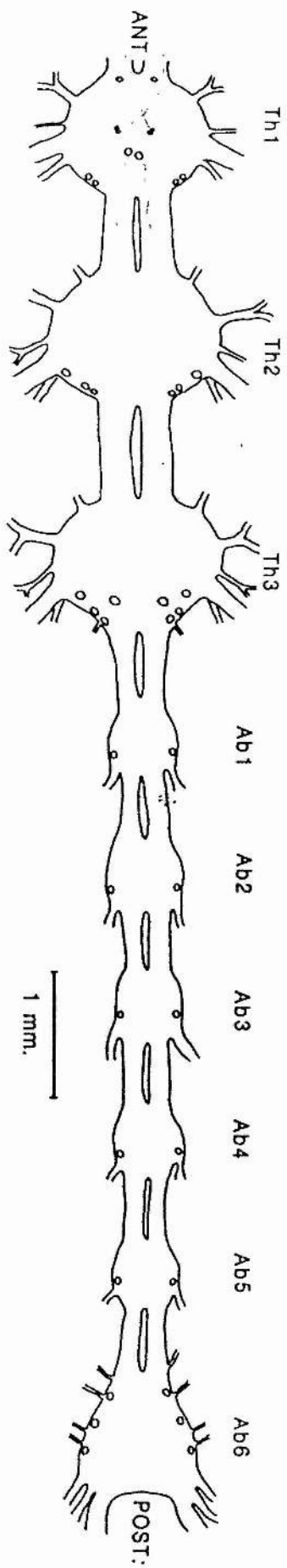


Figure 1.6, a and b Prothoracic ganglia of nymphal cockroaches (ventral view) after treatment with glyoxylic acid.

(a) 4 pairs of blue-green neurones are seen. At the posterior edge of the ganglion there are 2 pairs of neurones - the 2 cells on each side are practically superimposed. Calibration 100  $\mu$ m.

(b) 4 pairs of blue-green neurones are visible. In this case the most posterior pairs of cells in (a) are not seen, instead there are 2 very small pairs just above the large midline ones. Calibration 100  $\mu$ m.

**a**



**b**





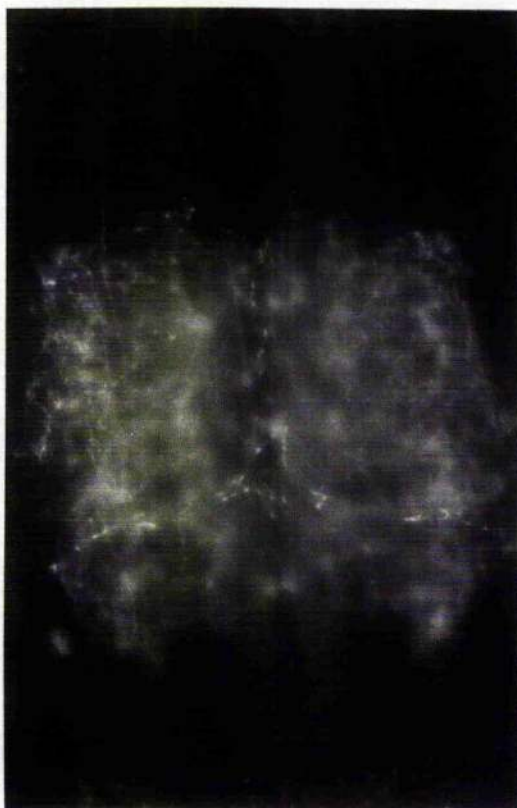
Figure 1.7, a and b 4th abdominal ganglion of a nymphal cockroach after treatment with glyoxylic acid. The two photographs are different focal planes of the same ganglion. All specific fluorescence was blue-green.

(a) Note the fluorescent fibres in the anterior connectives; and the dense varicose fibre network throughout the neuropile. Calibration 50  $\mu$ m.

(b) Different regions of the neuropile are now in focus. The cell bodies are more clearly seen - note the axon emerging from the cell on the left side. Some of the fibres can be seen to pass straight through the ganglion. ( $\triangleright$ ), cell body and axon; ( $\blacktriangleright$ ), through fibres. Calibration 50  $\mu$ m.

In both (a) and (b) it may be seen that the fibres in the connectives are non-varicose.

*a*



*b*

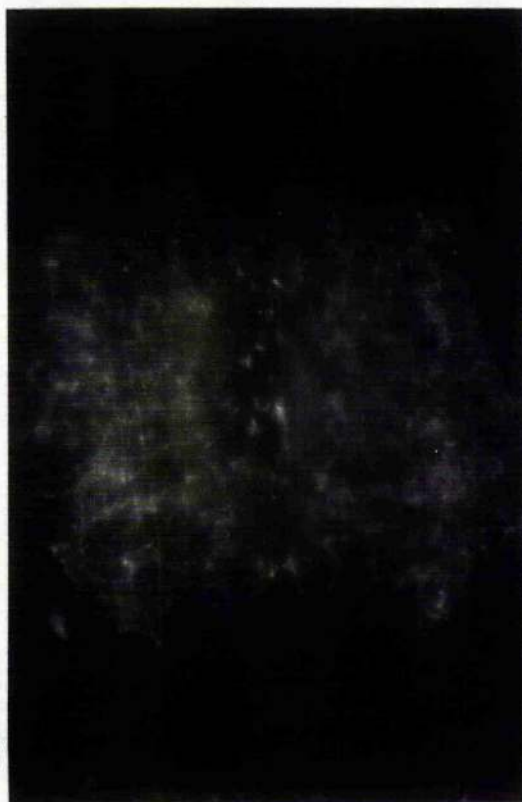


Figure 1.8 1st abdominal ganglion of a nymphal cockroach (ventral view) after treatment with glyoxylic acid. The ganglion had been exposed to bright light for several minutes before the photograph was taken. It may be seen that the fibre network is becoming fairly indistinct. Hence it is important to photograph the ganglia immediately, before carrying out a more detailed examination. Note, the colour of the specific fluorescence observed under the microscope was blue-green. Calibration 50  $\mu$ m.

Figure 1.9 1st abdominal ganglion of a nymphal cockroach (ventral view) after treatment with glyoxylic acid. In this photograph the bilaterally symmetrical nature of the innervation in the two halves of the ganglion is illustrated in the positions of (a) the fluorescent cell bodies (C); (b) the fluorescent nerve axons ( $\blacktriangleright$ ); and (c) the regions of intensely fluorescent varicosities down the centre of the ganglion (\*). Calibration 50  $\mu$ m.

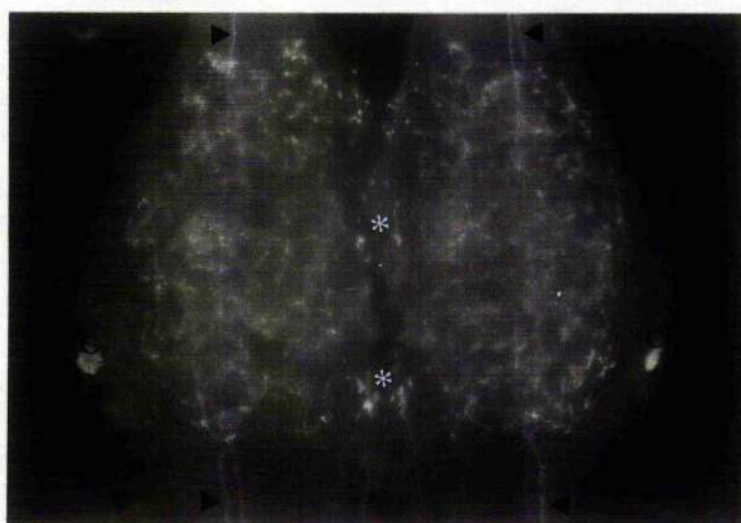
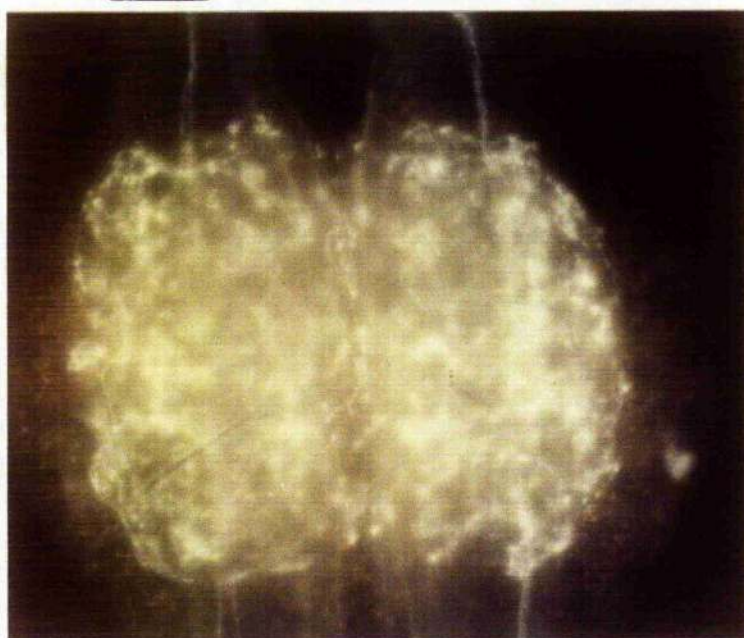


Figure 1.10 6th abdominal ganglion of a nymphal cockroach (ventral view) after treatment with glyoxylic acid. As with the smaller abdominal ganglia the fluorescent fibre network is present throughout the neuropile. Due to the way in which the dorso-ventral flattening occurred during air-drying, the cell bodies (▷) are only visible on the left side. Note how the fibres from the connectives quickly become lost in the neuropile. Calibration 50 µm.

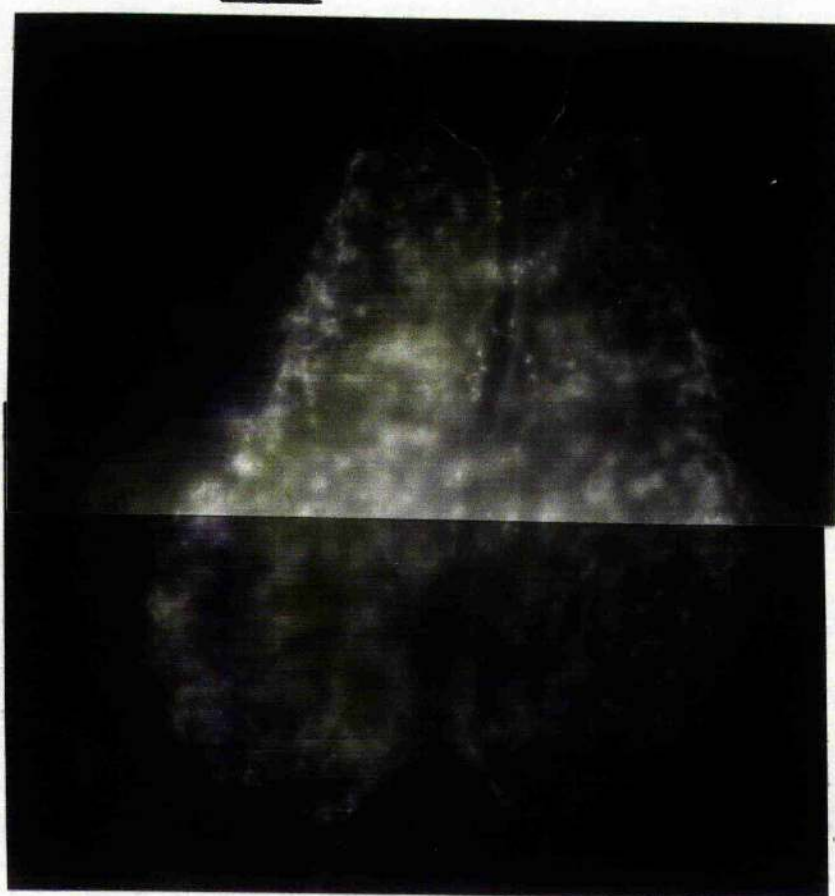


Figure 1.11 Mesothoracic ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The most anterior edge of the ganglion is shown. As in the abdominal ganglia the varicose fibre network is very dense. Note that the fibres in the anterior connectives very quickly become lost in the ganglionic neuropile and that fibres in identical positions in each connective may be seen (►). All specific fluorescence was blue-green. Calibration 50  $\mu$ m.

Figure 1.12 Metathoracic ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The right lateral portion of the ganglion is shown. Again the varicose fibre network is seen to be dense, with the fibres around the edge of the ganglion being the most distinct since the tissue is at its thinnest there. All specific fluorescence was blue-green. Calibration 50  $\mu$ m.







Figure 1.13 Prothoracic ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. Note that there are two nerve fibres (one in each connective) which may seem to pass straight through the ganglion (▷). (C), cell body. All specific fluorescence was blue-green. Calibration 100  $\mu$ m.

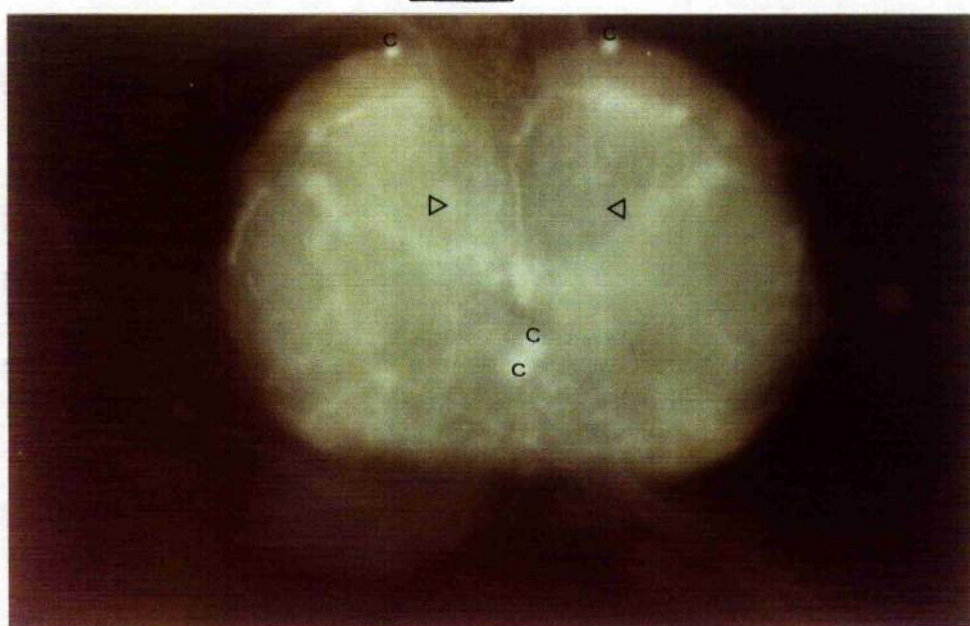


Figure 1.14 Prothoracic ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The anterior edge of the ganglion is shown. Note that the fibre positions in one connective are bilaterally symmetrical with those in the other (►). The most anterior pair of cell bodies is clearly visible (C), cell body. Calibration 50  $\mu$ m.

Figure 1.15 6th abdominal ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The most anterior half of the ganglion is shown. Note that as in the first ganglion of the ventral nerve cord (Fig. 1.14), the last one also has symmetrical axons in the connectives. On comparison, it may be seen that two of the pairs of symmetrical fibres (►) are in the same position in the two photographs. These same fibres could often be followed through one or two of the thoracic and all of the abdominal ganglia. This suggests that these fibres originate from neurones in the brain or SOG. Note too the intensely fluorescent varicosities (\*) located down the midline of the ganglion. These were seen in all the ventral nerve cord ganglia (see for example Figs. 1.7 and 1.9. (C), cell body. Calibration 50  $\mu$ m.



Figure 1.16 6th abdominal ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The posterior half of the ganglion is shown. Note that each cercal nerve (►) contains a varicose fibre (▷; clearest on the right hand side). All specific fluorescence was blue-green. Calibration 50 µm.

Figure 1.17 1st abdominal ganglion of a nymphal cockroach (ventral view) after glyoxylic acid. The animal had been pretreated with an injection of reserpine. Note there is no fluorescent fibre network in the neuropile region; only one or two faint fluorescent fibres are visible in the connectives (▷); and only one cell body (C) in the ganglion. Calibration 50 µm.



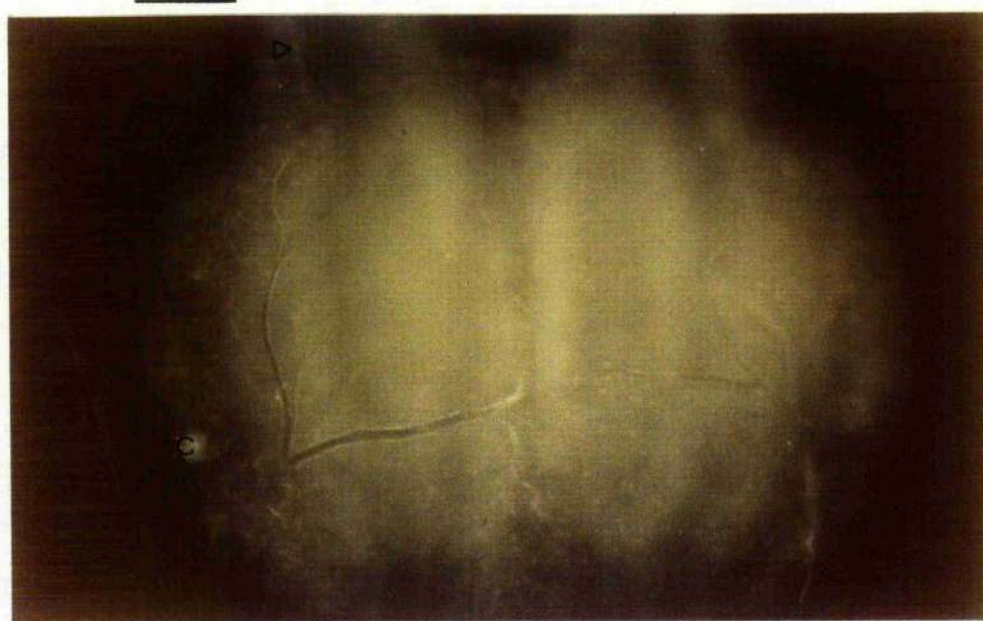


Figure 1.18 Suboesophageal ganglion of an adult cockroach (ventral view) after glyoxylic acid. A total of 23 cell bodies may be seen within the ganglion (see below). All specific fluorescence was blue-green. Calibration 100  $\mu$ m.

Figure 1.19 Diagram of the above ganglion (ventral view) showing the positions of the blue-green fluorescing neurones, and the main peripheral nerves and circumoesophageal connectives which are not visible in the photograph. COC, circumoesophageal connective. PC, posterior connectives. Calibration 100  $\mu$ m.



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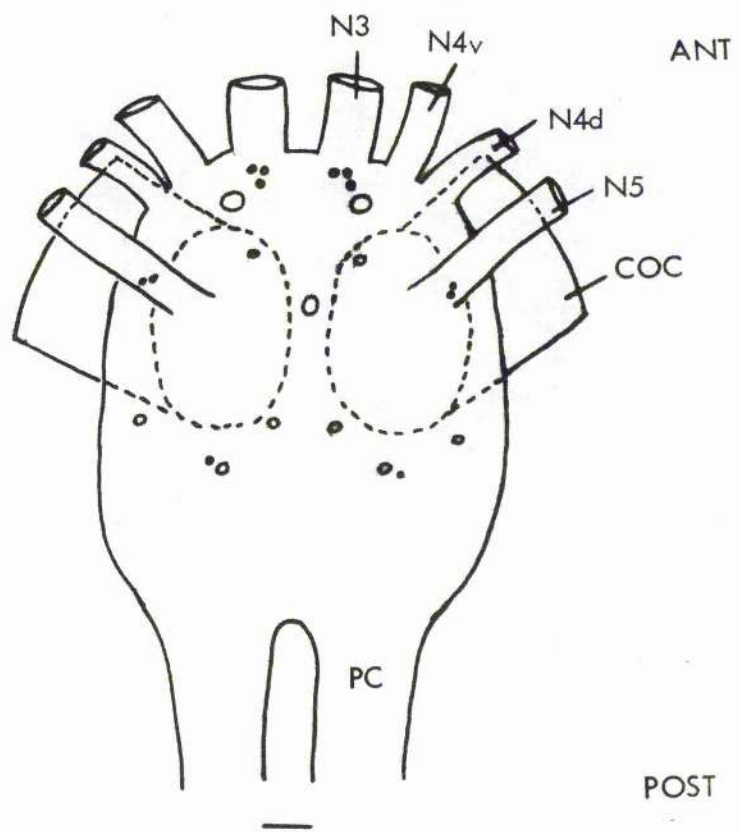




Figure 1.20 Suboesophageal ganglion of an adult cockroach (ventral view) after glyoxylic acid. Anterior end of the ganglion is shown. Note the unpaired midline cell (▷) and the bilaterally symmetrical fibres (►) which originate from the brain and the large pair of anterior neurones (\*), which are located on either side of it. Calibration 100 µm.

Figure 1.21 Suboesophageal ganglion of an adult cockroach (dorsal view) after glyoxylic acid. The fluorescent unpaired midline cell is seen in the centre; on either side are the varicose fibres (►). (▷). trachea. Calibration 50 µm.

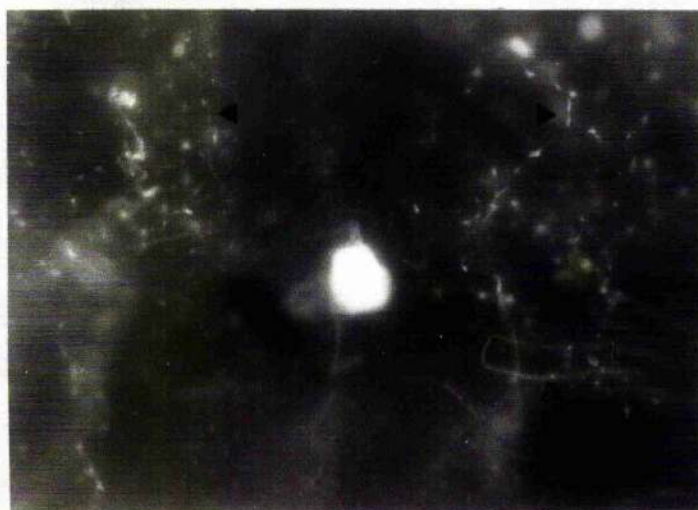


Figure 1.22 Suboesophageal ganglion of an adult cockroach after glyoxylic acid. The cell body of the unpaired midline neurone is seen at the top of the photograph; its axon leaves dorsally and quickly splits into 2 main branches (▷). Calibration 100  $\mu$ m.

Figure 1.23 Salivary duct nerve of an adult cockroach after glyoxylic acid. The nerve has doubled back on itself; it contains one strongly (▶) and one weakly (▷) fluorescing axon. (- -), edge of nerve. Calibration 25  $\mu$ m.

Figure 1.24 Suboesophageal ganglion of an adult cockroach (ventral view) after glyoxylic acid. Posterior half of the ganglion is shown. An axon may be seen emerging from the larger of the lateral pair of cells on the right half on the ganglion (▶). Calibration 100  $\mu$ m.



Figure 1.25 Suboesophageal ganglion of an <sup>adult</sup> cockroach (left lateral view) showing position of connectives and main peripheral nerves. PC, posterior connective; COC, circumoesophageal connective; LbN, labial nerve; MxN, maxillary nerve; MdN, mandibular nerve; CH I, II, substomodeal commissures; LN, labral nerve; FC, frontal connective.

Figure 1.26 Suboesophageal ganglion of an adult cockroach after glyoxylic acid. The edge of the ganglion and part of one of the circumoesophageal connectives and tritocerebrum is shown. Note that fluorescent fibres pass between the ganglion and the connective; the frontal connective and part of the tritocerebrum are highly fluorescent; and a fluorescent fibre is present in substomodeal commissure I (►). SOG, suboesophageal ganglion; TR, tritocerebrum. the rest of the labelling is as in Fig. 1.25. Calibration 100 µm.

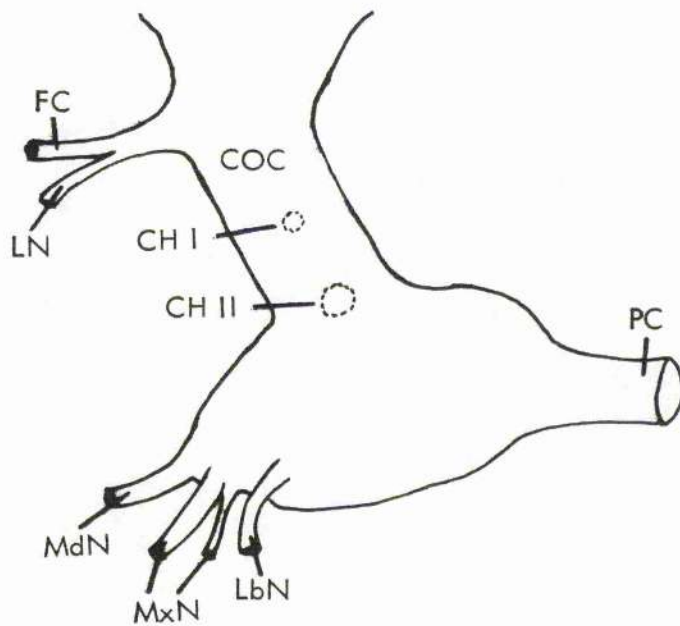
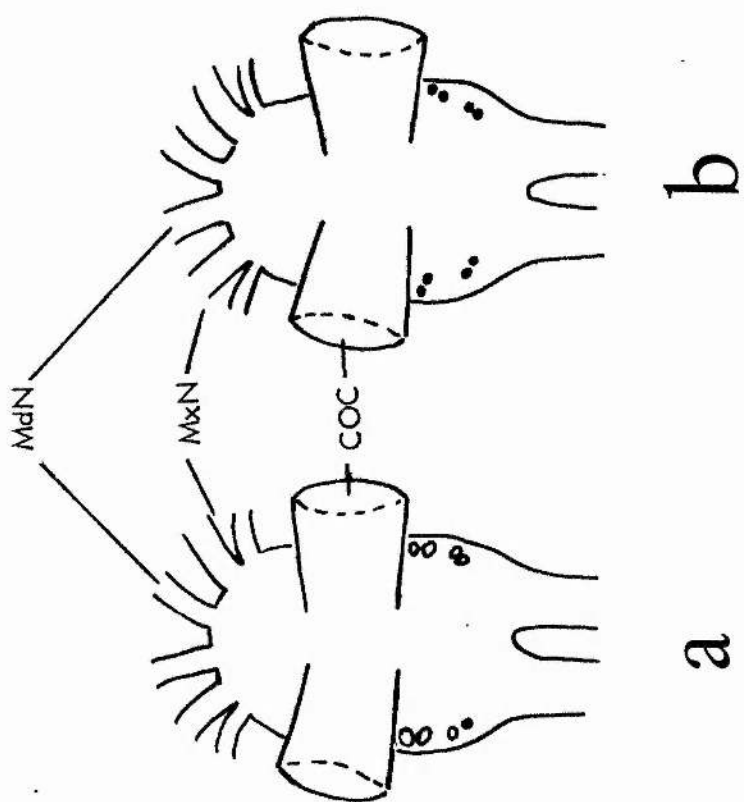


Figure 1.27, a and b Schematic drawing of the dorsal surface of an adult suboesophageal ganglion showing the positions of putative serotonin-containing neurones after (a), glyoxylic acid and (b), immunocytochemistry (Bishop and O'Shea, 1983). Labelling as in Fig. 1.25. In (a), nialamide had been added to the pre-glyoxylic acid incubation medium.





DISCUSSION

The results show that it is possible to locate catecholamine-containing neurones and fibres in whole mount cockroach ganglia. Klemm (1982) has also reported the development of a modified GA-formaldehyde technique for use in insect brain. It was similar, however, to the de la Torre and Surgeon modification (1976) in that the brain required cryostat sectioning. He also included a step in which the specimens were incubated with formaldehyde vapour for 1.5-2 hours. The technique developed in this study (a modification of Stuart, 1981) involves no sectioning and no incubation with vapour and therefore allows a much quicker and accurate determination of cell body position.

The initial experiments on adult ganglia (which used the leech GA protocol) were totally unsuccessful. The main reason for this, was probably that the GA had not penetrated far enough into the ganglia to react in the appropriate cells. It is well documented that the insect CNS, unlike that of many other invertebrates, is relatively insensitive to most applied drugs since it has an extremely effective blood-brain barrier (Treherne and Pichon, 1972). To diminish this problem it was decided to use nymphal cockroach ganglia which are smaller (thus reducing diffusional distances) and have a less well developed neural lamella, perineurium, and glial network. Also, as with any fluorescence technique, there is the problem of background autofluorescence which can obscure quite a lot of detail. Again this problem is reduced in the smaller, thinner, nymphal ganglia.

The leech protocol for the technique was modified for both nymph and adult cockroaches (cf. Tables 1.4, 1.5 and 1.6) to give greater GA penetration, and this resulted in good fluorescent preparations for both groups. It was still only in the nymphal preparations, however, that the intricate fluorescent fibre networks could be seen.

It was also noted that there was a difference in the length of time the specific fluorescence lasted in the cockroach and leech preparations. Leech slides that were several months old still showed very clear fluorescence in both the cell bodies and fibres. With the cockroach specimens, however, the fluorescence started to become diffuse as early as 24 hours after they were prepared. The cell bodies could remain clear for several days, even weeks, but the fibres, especially those present in the ganglia, soon became obscured in the general background fluorescence. Most of the cockroach specimens were pretreated to aid the penetration of the GA. And it was noted that, in general, the harsher the pretreatment the shorter the period of time in which the ganglionic fibre network was crisp and clear. The GA solution used for the cockroach contained four times the original concentration of GA. To counteract the increase in the solution's osmolarity the concentration of both the sucrose and the phosphate were halved. This did give more consistent results but was noted to destabilize the fluorescence. In fact, monobasic phosphate alone with the GA gave very good tissue fluorescence but the fibres networks very rapidly became totally indistinct.

Other factors that were found to affect the fluorescence

included:

(i) The oven incubation time. Below 5 min the fluorescence was weak, whereas above it the background fluorescence became intense and tended to mask the specific fluorescence.

(ii) The temperature of the air used in the blow drying stage. This had to be cool. Hot air gave specimens with a diffuse fluorescence.

(iii) The rapidity of the cool blow drying stage. If this was too slow, due to the blower having an insufficient air flow, the fluorescence was again diffuse. Specimens could, however, be left under the dryer for longer than the stated time, hence allowing several slides to be processed through the following stages together.

To be absolutely certain of the identity of a fluorophore it is necessary to examine the fluorescence by the technique of microspectrofluorometry. This technique is based on recording both the emission and the excitation spectra of the fluorophores. The spectra obtained are directly related to the molecular configuration of the fluorescent compounds, and hence would allow dopamine, noradrenaline and serotonin to be distinguished from other biogenic amines. The excitation/ emission maxima for dopamine and noradrenaline are 415 nm/ 475 nm (fluorophores in their neutral quinoidal form); and 330 nm and 375 nm/ 460 nm (fluorophores in their acidic non-quinoidal form; for serotonin the values are 375 nm/ 520 nm (serotonin only has one form). Dopamine and noradrenaline can be distinguished from one another by

treatment of the specimens with hydrochloric acid vapour; the isoquinoline derivative formed from noradrenaline becomes fully aromatic, and so the spectral properties change (Lindvall and Björklund, 1974; Björklund, Falck and Lindvall, 1975; Lindvall, Björklund and Svensson, 1974; Klemm, 1976). Unfortunately the apparatus required to carry out this analysis was not available. However, earlier experiments have shown that the only biogenic amines that are present in significant amounts in the cockroach nervous system are dopamine, noradrenaline, octopamine and serotonin (see Table I.2). Octopamine is non-fluorescent, serotonin gives a yellow-orange fluorescence and so the blue-green fluorescence is very probably due to either dopamine or noradrenaline.

Only a few fluorescence histochemistry studies in insects have included microspectrofluorometric analysis along with the additional experiments required to differentiate dopamine and noradrenaline. Klemm and Axelsson (1973), working on the cerebral ganglia of locusts, found that most of the cells gave spectra characteristic of dopamine. It was only in the pars intercerebralis that a few noradrenaline-containing cells were detected. (See also Klemm and Falck, 1977). In the medulla of the optic lobe these authors found that in the first instar the second layer of this structure produced spectra consistent with a noradrenaline fluorophore, whereas in the adult dopamine was identified. One possible explanation is that during development the number of dopaminergic terminals greatly increases in this area and this produces masking of the noradrenergic

fluorescence in the adult. They also reported that the alpha and beta lobes and the central body complex produced spectra which suggested that these structures either contained both dopamine and noradrenaline fibres, or that both monoamines appeared in the same fibre (see Björklund, Ehinger and Falck; 1972). Björklund, Falck and Klemm (1970) were unable to detect noradrenaline using microspectrofluorometric analysis in the thoracic ganglia of Trichoptera, although they did find it using a chemical determination, with its concentration being about tenfold less than dopamine. It is possible that the noradrenaline was contained within more weakly fluorescent fibres which could not be analysed using the fluorescence microspectrograph; or that, as before, the noradrenaline and dopamine are found in the same fibres, except that in this case the concentration of dopamine is such that it dominated the spectral recordings. Noradrenaline fibres have been detected in the cerebral ganglia of Trichoptera (Klemm and Björklund, 1971), but as with the locust ganglia, these are intermingled with dopamine-containing ones. No isolated noradrenaline-containing neuropilar structures were found. Again no noradrenaline-containing cell bodies were found, although these could have been amongst the low fluorescence intensity ones that could not be analysed.

Due to this lack of positive evidence for the presence of specific noradrenergic neurones, Evans (1980b) suggested that although a small group of noradrenergic neurones could exist, it is also possible that the small quantity of noradrenaline detected in the insect nervous system could be

a metabolic artefact. He pointed out that this would occur if any dopamine were taken up into octopaminergic neurones. These cells contain the enzyme tyramine- $\beta$ -hydroxylase which can also catalyse the conversion of dopamine to noradrenaline (Evans, 1980b). From the above evidence, therefore, it is very likely that most, if not all, of the blue-green fluorescent cells and fibres located in this study are dopaminergic.

No neurone with a yellow-orange fluorescence characteristic of serotonin were seen in the nymph preparations; a few very rapidly fading yellow-orange fluorescent cells were seen in nialamide treated adult suboesophageal ganglia (SOG; see below). Having carried out preliminary experiments in the leech it was known that serotonin could be detected by this technique (see also Stuart, 1981; Lent, 1982). In 1974, however, Lindvall and Björklund, working on GA-treated rat brain specimens, reported that indolamine-containing neurones only gave a brownish-yellow, weak and variable fluorescence. De la Torre and Surgeon (1976) found that serotonin fluorescence was very weak and labile in rat and mouse brain tissue but stable and bright in dog and monkey brain. With regard to insect preparations, Klemm (1982) was unable to detect serotonin using his modified GA-formaldehyde technique. It is worth noting that this difference in the reactivity of the serotonin and the photostability of its fluorescence between leech, insect and vertebrate preparations has also been found with the Falck-Hillarp technique; as above the leech serotonin fluorescence is bright yellow and stable for several minutes (Marsden and



Kerkut, 1969; Lent Ono, Keyser and Karten, 1979); in vertebrate and insect preparations the fluorescence is brownish yellow, weak (and often absent), and fades within a few seconds (Dahlström and Fuxe, 1964a; Frontali and Norberg, 1966; Frontali, 1968; Klemm, 1983). The reason for this difference is unknown. Perhaps it could be due to differences in the chemical make up of the nervous tissue, or even just to the fact that leech neurones contain a much higher concentration of serotonin. It has been estimated that the serotonin concentration in the Retzius and ventral lateral neurones is 6 mM (Rude et al., 1969) and at least a 100 mM (Lent et al., 1979) respectively; for an identified Aplysia neurone it was estimated at 0.94 mM (Kehoe and Marder, 1976). Because of the problem of poor results with serotonin fluorescence, the use of fluorescence techniques to localize this compound are being abandoned, at least in the insect, in favour of immunocytochemistry (Bishop and O'Shea, 1983; Klemm and Sundler, 1983).

The nialamide DOPA and colchicine injections (nymphs) and the nialamide incubation (adult SOG) were used to increase the fluorescent yield. Nialamide is a monoamine oxidase inhibitor, whereas DOPA is a catecholamine precursor. Hence, if these compounds were taken up in sufficient amounts, there would be an increase in the catecholamine content of the appropriate neurones. Since DOPA itself forms highly fluorescent derivatives in the GA reaction its conversion would not be required. The result would be an increased fluorescence in both the cell bodies and fibres. In the case of the nymph preparations very little increase was seen.

With the SOG, the catecholamine fluorescence was likewise little affected, but yellow-orange fluorescent cells were consistently seen only in the nialamide treated preparations. In Falck-Hillarp and GA treated preparations of vertebrate brains it has been found that monoamine oxidase inhibitors have little effect on the catecholamine fluorescence, whereas they either significantly increase the fluorescence of, or bring about the appearance of, serotonin-containing neurones (Dahlström and Fuxe, 1964a; Lindvall and Björklund, 1974). This enhancing effect of nialamide has also been seen with other Falck-Hillarp treated insect preparations (Klemm and Axelsson, 1973; Elofsson and Klemm, 1972). The enhancing effect of nialamide in insects is in fact quite surprising, since it has been shown that the level of monoamine oxidase (MAO) activity in the brain of Apis mellifera (Evans and Fox, 1975) and the central nervous system of Drosophila and Periplaneta is very low (Dewhurst et al, 1972; Beeman and Matsuura, 1974). Richter and Rutschke (1977), however, did report MAO activity in certain regions of the brain of Periplaneta. The enzyme was dopamine specific, having no action on serotonin and was inhibited by pargyline (a vertebrate MAO inhibitor). Houk and Beck (1978) also found MAO activity in the brain of the European corn borer, Ostrinia nubilalis. This activity was sensitive to the MAO inhibitors tranylcypromine and nialamide. The reason for the differing reports could be due to the method of detection employed. Those workers who found little or no MAO activity used biochemical measurements, whereas the positive results were obtained using histochemical techniques. This contradiction



and the positive effect of nialamide on serotonin fluorescence clearly indicate that more work must be carried out before anything concrete can be said about MAO levels in insect CNS. In the case of treatment with DOPA (with Falck-Hillarp preparations), it has been found that the specific catecholamine fluorescence in vertebrate preparations is little affected (Dahlström and Fuxe, 1964a); in insects it is possible to follow the catecholamine-containing fibres for longer distances (Klemm, 1976).

As stated above it was only with the adult SOG preparations that nialamide had any effect on the serotonin fluorescence. This <sup>was</sup> probably due to the method of application of the drug. For the adults the nialamide was added to the pre-incubation saline; for the nymphs nialamide-DOPA was injected into the abdomen 2-3 days prior to experimentation. It is unknown how well the drugs penetrated the insect's blood-brain barrier and so it is possible that an ineffective concentration in the CNS was the cause of the lack of effect.

Some of the experiments on the nymphs included a pre-experimentation injection of colchicine. This drug interferes with axonal transport. Injection with this, therefore, would cause catecholamines to accumulate in the cell body and give increased fluorescence in this region. In general a small effect was seen. Limited access to the CNS could again have prevented the result from being more significant.

Reserpine is a Rauwolfia alkaloid which has been used to deplete the storage vesicles of catecholamines. The uptake

of noradrenaline, dopamine and serotonin into the storage vesicles is an active process which requires ATP (energy source) and magnesium ions (to activate the ATP-ase enzyme). Without the magnesium the stability of the catecholamine-ATP-protein-metal ion storage complex is markedly reduced. Reserpine is a magnesium chelating agent; and thus the uptake of catecholamine into storage vesicles is reduced. The result is that the catecholamine leaks out into the cytoplasm where it is broken down (Kruk and Pycck, 1979). For example, Sloley and Owen (1982) found that reserpine (5-100  $\mu$ g), injected into the abdominal haemocoel of adult cockroaches (Periplaneta americana), caused a dose dependent reduction in the nervous tissue levels of noradrenaline, dopamine and serotonin. An injection of 40  $\mu$ g produced almost maximal depletion; these minimal levels lasted for 3-5 days and were followed by a slow return towards control levels. In the case of fluorescence studies, the catecholamine depletion produced by this drug has been used by a number of workers to show firstly, that the fluorescence they have obtained is specific; and secondly, that it is due to the presence of a catecholamine (for example, Dahlström and Fuxe, 1964a and b; Falck, 1962; Falck and Owman, 1965; Frontali, 1968; Lindvall and Björklund, 1974). A significant decrease in the specific blue-green fluorescence of the nymph ventral cord ganglia following an injection of reserpine was seen in this study; thereby indicating the presence of either dopamine or noradrenaline. In general no fluorescent fibres were visible and the cells were much less distinct and often absent. Sloley and Owen

(1982) reported that the reserpinised animals became very lethargic. This behavioural change was also found with the nymphs in this study.

Only two pairs of peripheral nerves were shown to contain blue-green fluorescent axons. The pair which originated from the SOG are the salivary duct nerves. One very fine axon was seen to extend into the cercal nerve; it is not known whether it is a sensory or motor fibre, although it is known that this nerve is predominately sensory (Guthrie and Tindall, 1968). It is also possible, however, that its role is modulatory, as is found with peripheral octopaminergic neurones ( see Evans, 1980b, for a review).

Each ganglion contains an extensive network of biogenic amine-containing fluorescent fibres which originate from one or more of the following sources: (a) from the cells in that ganglion; (b) from cells in the brain; and (c) from cells in the ganglia situated either anteriorly or posteriorly or both. This type of extensive fibre network is also seen in leech ganglia, where the processes of only one pair of dopaminergic neurones ramify throughout the whole neuropile (see Lent, 1982; Rude et al., 1969). In the vertebrate CNS too, fluorescence histochemical studies have shown that the terminal field of the dopaminergic neurones in the striatum is extremely dense. Each axon has a prolific collateralization in which each branch contains numerous small varicosities. Andén, Fuxe, Hamberger and Hökfelt (1966) estimated the number to be close on half a million. Although at the moment little is known about the role of the dopamine (and noradrenaline) in the insect CNS, it is

probable that since the innervation has been shown to be very extensive, the biogenic amine-containing neurones must contribute to a substantial degree in the integration and processing of information.

From the photographs of the results (see for example Fig. 1.7) it is seen that each ganglion contains two types of blue-green fluorescent fibre; varicose and non-varicose. In general it is the fibres which pass straight through the ganglia that are the non-varicose ones. This result ties in with the fact that in vertebrates varicosities are known to be transmitter release sites, and in insects it is only in the neuropilar regions that synapses are made.

Although the dopamine released from the terminal varicosities may function at certain synapses as a classical neurotransmitter, there is also the possibility that at others its action may be modulatory. For example, in Aplysia, serotonin brings about dishabituation of the gill withdrawal reflex. It acts presynaptically, increasing the effectiveness of transmission across the sensory-motor synapse (Castellui, Kandel, Schartz, Wilson, Nairn and Greengard, 1980). Also in Aplysia, it has been shown that certain serotonin-containing neurones (the giant metacerebral cells) are involved in the control of feeding behaviour. The cells have an effect on certain buccal motoneurones both in the CNS, through conventional excitatory synapses, and in the periphery, by modulating the efficacy of transmission from the motoneurones (Weiss, Cohen and Kupfermann, 1975, 1978). Dopamine is also involved

in the control of the gill movements, although its action is confined to the periphery. Firstly it acts as a transmitter at the neuromuscular junction to bring about a characteristic type of contraction (Ruben, Swann and Carpenter, 1979). Secondly, it increases the efficacy of transmission of certain motoneurons at the neuromuscular junction (Ruben and Lukowiak, 1983).

Since from the photographs it is obvious that many of the varicosities do not occur at fibre terminals, it is worth considering whether or not these varicosities (as well as the terminal ones) may be transmitter release sites. In addition, there arises the possibility that some of the transmitter release may not be at conventional one-to-one chemical synapses. Work on vertebrate and other invertebrate nervous systems has produced evidence both for and against these suggestions and some of this is summarised in the following paragraphs.

#### Vertebrate CNS

In 1975, Bjorklund and Lindvall reported that not only the terminal axons, but also the dendrites of the substantia nigra pars compacta neurones possess an extensive dopamine-containing varicose fibre network. An ultrastructural study of the endings (Wilson, Groves and Fifkova, 1977) after 5-hydroxydopamine uptake showed that both labelled axons and dendrites containing synaptic vesicles form synapses 'en passage' in the substantia nigra; in some cases the postsynaptic element in a dendro-dendritic synapse was identified as also being dopaminergic. Apart from being

involved in the autoregulation of the dopaminergic neurones, there is also accumulating evidence that endogenous dopamine from the dendrites can modulate the inhibition of substantia nigra pars reticulata neurones elicited by GABA iontophoresis or striatal stimulation (Waszczak and Walters, 1986).

Several groups of investigators have examined the morphology of the cortical and other projections of monoaminergic neurones. Controversy, however, arose over the ultrastructure of the synapses. Descarries and his co-workers found that although the axonal varicosities have the appearance of classical synaptic boutons, less than 5% are connected to post-synaptic membranes (Descarries, Beaudet and Watkins, 1975; Descarries, Watkins and Lapierre, 1977; Leger and Descarries, 1977; Beaudet and Descarries, 1978). From these results it has been speculated that the aminergic systems are neuromodulatory in function; with the monoamine altering the responses of target cells to the classical neurotransmitters by means of a diffuse 'garden sprinkler' release (Kuffler, Nicholls and Martin, 1984; Dismukes 1977a, b and 1979; Moore and Bloom, 1979; Descarries et al, 1977).

The main problem with this hypothesis is that it is based on largely negative data, that is failure to visualize synaptic junctions in axon profiles labelled autoradiographically with tritiated amine. When using other techniques - namely, 5-hydroxydopamine uptake or immunocytochemistry involving anti-bodies against dopamine- $\beta$ -hydroxylase or serotonin -



it has been found that 40-80% of all labelled varicosities in the cortex and other brain regions establish synaptic contacts (Molliver, 1982; Molliver, Grzanna, Lidov, Morrison and Olschowka, 1982; Groves, 1980; Koda and Bloom, 1977). Most of the above studies involved the examination of random ultrathin sections in the electron microscope. This results in some synaptic contacts being missed because they are out of the plane of section. After taking account of this, Molliver et al (1982) concluded that all noradrenaline varicosities in the rat cortex form synaptic contacts.

It appears therefore that the autoradiographic studies mentioned above grossly underestimated the number of synaptic contacts and in reality, the formation of synapses by monoaminergic axonal varicosities is the general rule and not the exception.

#### Vertebrate peripheral nervous system

The situation is totally different in the vertebrate peripheral nervous system where it has been known that 'non-synaptic' release, (that is release that is not confined to a terminal bouton and/or action that is not confined to a specialized region of the one post-synaptic cell), occurs from the postganglionic neurones of the autonomic nervous system (Merrilles, Burnstock and Holman, 1963; Cunnane, 1984; Kandel, 1985). The axon terminals of these neurones are highly branched and consist of varicosities and thin intervaricose segments. Although the varicosities are transmitter release site, there are no pre- or post synaptic specializations and the gap between the varicosity and the



tissue may be as wide as 2  $\mu$ m. This results in transmitter action being wide spread. The list of putative transmitters at these synapses has grown in recent years and there is also evidence for the co-existence of transmitters as well as modulation of their release (Kandel, 1985; Burnstock, 1981 and 1982).

#### Invertebrate nervous systems

'Non-synaptic' release has also been found in invertebrates. It is known that the swimming activity of leeches is determined by the general level of serotonin in the CNS (the serotonin is acting as a neurohormone; see Kristaun and Nusbaum, 1983, for a short review). In the periphery of the locust, octopamine is released from blind ending neurosecretory terminals of certain cells, where it has a potentiating effect on neuromuscular transmission Hoyle, 1975; Hoyle and Barker, 1975; Hoyle et al, 1974; Hoyle et al, 1980; Evans and O'Shea, 1977 and 1978; O'Shea and Evans 1979; Evans 1981, 1984a and b; see also pps 40, 41, 158 and 159). In the lobster and crayfish, both serotonin and octopamine are released from neurosecretory terminals of peripheral nerves into the circulation where they prime the muscle to respond more vigorously to the junctional transmitters (Kravitz, Beltz, Glusman, Goy, Harris-Warrick, Johnston, Livingstone, Schwarz and Siwicki, 1983 and 1985; Fischer and Florey, 1983). Finally, in crustaceans the stomatogastric ganglion receives a substantial input from other ganglia via the stomatogastric nerve. A fair number of putative transmitters, including the biogenic amines, have been located in these incoming fibres and each substance is

known to modulate the output of the network of pyloric neurones in a specific manner. It is thought that some of these substances may be released at some distance from their final site of action (Marder and Hooper, 1985; Marder, 1985 and 1986).

From the above it is clear that each biogenic amine could act in the following ways in the CNS of the cockroach: as a neurotransmitter whose release affects postsynaptic cells in the 'classical' way (that is produces an EPSP or an IPSP) and/or as a substance that modulates other inputs to a neurone, again either in a directed or a more diffuse fashion, but has no effect itself on the membrane potential of the postsynaptic cell(s). This study has resulted in the identification of a number of catecholamine-containing cells in the ventral nerve cord of the cockroach. Certain of the cells (for example, the medial pair in the first thoracic ganglion) are large enough for intracellular studies. Hence in the future it should be possible to characterize these cells both anatomically and electrophysiologically. Electron microscopy could be carried out to discover whether or not the non-terminal boutons are potential transmitter release sites. Follower cells could also be located. These experiments have thus provided a starting point from which it should be possible to ascertain at least part of the role played by the catecholamines in the cockroach central nervous system.

SECTION II

THE AXONAL PROJECTIONS OF THE PROTHORACIC (T1) CATECHOLAMINE  
CONTAINING NEURONES.

INTRODUCTION

In the prothoracic ganglion 6 pairs of blue-green fluorescing nerve cell bodies have been identified (Fig.1.5). In some of the nymph preparations blue-green fluorescing fibres could be seen emerging from a few of these cells, but since the fibres quickly became lost in the background fluorescence of the neuropile it was not possible to tell if the cells were local interneurons or if they projected into other ganglia. There are two ways to ascertain the axonal projection of a cell: (i) intracellular injection of a dye into the cell body; and (ii) retrograde transport of a dye up the axon to the cell body. Measurements from photographs of adult preparations indicated that the diameter of the large medial pair of the cells (Fig.1.5) is about 20-25  $\mu\text{m}$ , all the others are below 15  $\mu\text{m}$ . Hence it was considered that, apart from the large medial pair, all the other cells present in the prothoracic ganglion (and the rest of the ventral nerve cord) were too small to be readily and repeatably identified for intracellular injection. To determine possible axonal projections of all the cells in the prothoracic ganglion it was decided to use dye infusion in combination with glyoxylic acid (GA). For the larger medial pair intracellular dye injection in combination with GA could then be used to provide a more detailed picture of these cells' morphology.

It has been shown that in the prothoracic ganglion only the anterior and posterior connectives contain fluorescent fibres (see section 1) and hence if any fluorescent cells

project beyond the ganglion their axons must be present in one or more of the connectives. To ascertain this hexaminecobaltic chloride dye infusion was used in combination with GA. Axonal iontophoresis of cobalt chloride in the insect nervous system was first introduced by Pitman, Tweedle and Cohen (1973). It has since been found that the current is unnecessary with the dye travelling well using simple retrograde axonal diffusion. All fluorescing cells are paired, and so if dye is infused into only one of the connective pairs it should be possible to see one black stained cell and a paired fluorescing one.

The axonal projections of the large medial pair may be revealed by the above but little detail of the cells' morphology will be obtained with <sup>the</sup> retrograde infusion method. To determine detailed morphology intracellular dye injection is required. Lucifer Yellow is a superfluorescent dye, developed at the National Institutes of Health (Stewart, 1978). The dye has been used to ascertain the morphology and/or electrophysiology of neurones in several insect species including fruitflies (Thomas and Wyman, 1983; Thomas, Bastiani, Bate and Goodman, 1984), cockroaches (Ernst and Boeckh, 1983), locusts (Robertson and Pearson, 1982 and 1983; Rowell and Pearson, 1983) and grasshoppers (Bate and Grunewald, 1981; Goodman and Spitzer 1979 and 1980; Thomas et al, 1984). It can be viewed both in living tissue and after fixation. The fixative used most often following Lucifer Yellow injection is 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). This is fairly close to the solution used in the GA incubation - with the formaldehyde

being replaced with GA - and so it was considered that the GA solution could replace the normal fixative. Also it was possible to view the dye using the same Leitz fluorescence filter block as used with the GA processed specimens. The aim was, therefore, to inject one the catecholamine-containing medial cells with Lucifer Yellow and follow this with the GA technique. This would result in one yellow and one blue-green fluorescing cell paired at the midline.

MATERIALS AND METHODS

Since the main problem with the intracellular injection was filling the correct cell, preliminary experiments were performed to show the distribution of the neurones on the ventral midline region of the prothoracic ganglion.

Toluidine Blue Staining of the Prothoracic Ganglion

The procedure used here follows that described by Altman and Bell (1973) and Altman (1980). The compositions of the staining and the differentiator/fixative solutions are given in Table 1.7.

Freshly dissected prothoracic ganglia were immersed in warmed stain in a small glass vial. Staining was carried out for 10 min in a 50°C oven. The ganglia were then transferred directly to Bodian's No. 2 fixative. This solution very quickly became saturated with the stain and so was changed several times in the first few minutes, until the solution stayed almost colourless. The ganglia were dark blue at this stage. Differentiation was continued until the cell bodies were clearly visible but the nerve roots nearly white. Differentiation was stopped slightly before the desired result since dehydration causes a little more stain to be lost. The preparations were dehydrated in two changes of 90% ethanol (5 min each), and two changes of absolute ethanol (10 min each). They were then cleared and mounted in creosote, observed using the Leitz Dialux 20 microscope and photographed with the Wild Photoautomat system on Ilford FP4 125 ASA film (see Table 1.4 for developing procedure).



TABLE 1.7

COMPOSITION OF THE TOLUIDINE BLUE STAINING SOLUTION

Toluidine Blue	1g
Borax ( $\text{Na}_2\text{B}_4\text{O}_7$ )	6g
Boric Acid	1g
Distilled Water	100 ml

COMPOSITION OF THE DIFFERENTIATOR/ FIXATIVE SOLUTION

Bodian's No. 2 fixative (Bodian, 1937):

Formalin	5 ml
Glacial Acetic Acid	5 ml
80% Ethanol	90 ml

Hexamminecobaltic Chloride Dye Infusion

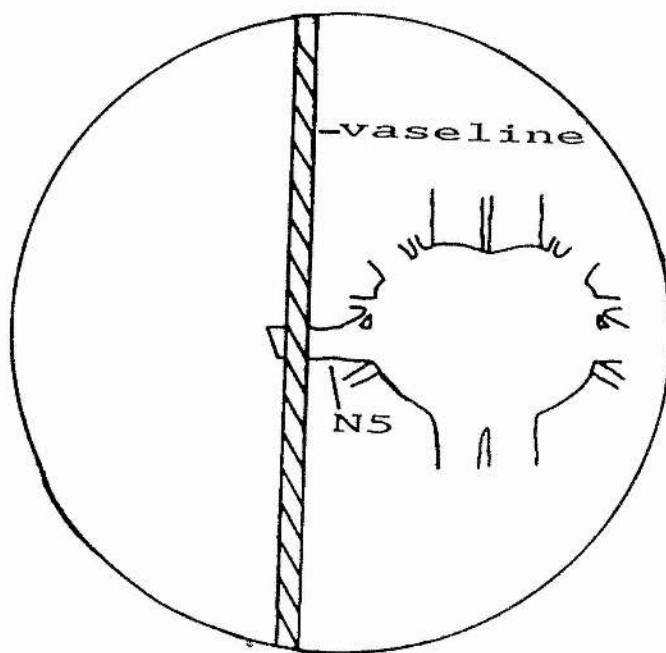
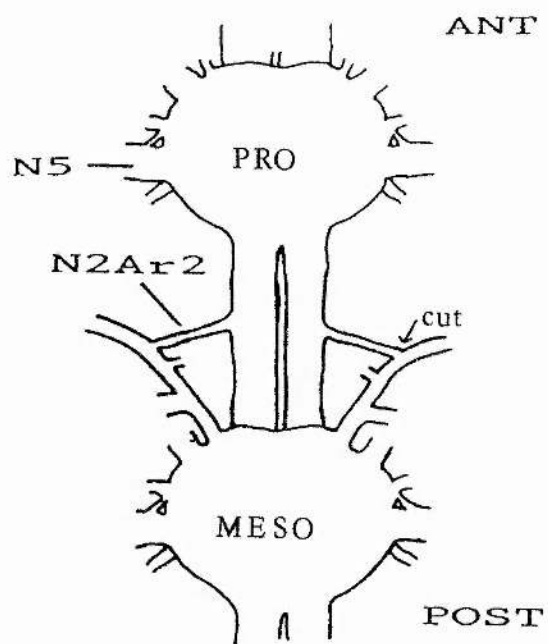
In order to show up some of the fairly large cell bodies which were in the same region of the prothoracic ganglion as the fluorescent medial pair, hexamminecobaltic chloride dye infusion into nerve 5 and mesothoracic nerve 2Ar2 was carried out (Fig.1.28). This would aid location of the medial neurone pair for the intracellular injection.

The thoracic ganglia were exposed as described previously (Section I). For the nerve 5 infusions the fifth prothoracic nerve trunk was exposed as far as the trochanter by cutting away the cuticle of the coxa. The nerve was cut, the surrounding tracheae gently pulled away, and then it was carefully lifted free from the muscle. The ganglia, and nerve, were excised as before.

The mesothoracic nerve 2 infusions. The mesothoracic nerve 2s divide into two main branches (2A and 2B) soon after they emerge from the ganglion. Branch 2A further divides and one of these branches fuses with the anterior ipsilateral interganglionic connective (Fig. 1.28). The thoracic ganglia were excised as described before (Section I), except that when the peripheral nerves were being cut the branches of the N2Ar2s to the connectives were cut as close as possible to their exit point from the main nerve.

Figure 1.28 Schematic diagram of the pro- and mesothoracic ganglia of a cockroach to show the position of prothoracic nerve 5 (N5) and mesothoracic nerve 2Ar2 (N2Ar2). PRO and MESO, prothoracic and mesothoracic ganglia.

Figure 1.29 Dye infusion set up for a prothoracic nerve 5 preparation. When complete, the ganglion is covered with a small piece of saline soaked filter paper; the cut end with cobalt solution soaked filter paper. The petri dish lid is placed in a small plastic box which is kept at 4°C for 18 hr.



The dye infusion was set up as follows (Fig. 1.29). Using a syringe, a thin wall of vaseline was laid across the surface of the lid of a small plastic petri dish. An electrically warmed wire was used to melt the vaseline so it formed a tight seal with the dish surface. A small drop of saline was placed on both sides of the wall and the preparation transferred into one of these. The nerve into which the dye was to be infused was laid across the wall into the other saline drop. Only the very tip of the nerve was held to prevent damage to the nerve fibres. The part of the nerve lying over the vaseline was dabbed dry with a tissue, a vaseline strip laid over it and melted down with the wire. Care was taken to ensure that the nerve was not touched by the wire. To maximise the surface area of the fibre endings the end of the nerve was cut obliquely near to the vaseline. Then, to ensure that all the endings were open the saline was dried up and a drop of distilled water containing 1% Triton x100 placed there for five minutes. This was then dried up and replaced by a drop 100mM hexaminecobaltic chloride solution. A small piece of filter paper was placed over the solutions on both sides and the petri dish put in a small plastic box containing moist tissue paper to keep the environment humid. This was then put in the refrigerator and kept at 4°C for 18 hrs.

The preparation was removed from the dish from the saline side by melting the vaseline and carefully pulling the nerve through. It was washed in saline and then immersed in about 5ml of saline containing one small drop of ammonium

sulphide solution. This was added to the saline immediately before use since it precipitates out on prolonged exposure to air. The preparation was left in the solution for about 10 mins to allow the precipitation of ammonium sulphide to occur. The cell bodies and fibres that had been filled become black. The tissue was washed in saline for 5 mins, transferred to a 1:1 solution of saline and 70% ethanol for 10 mins and finally placed in 70% ethanol in saline. The preparation could be stored in the refrigerator for several days at this stage if necessary.

Preparations were observed and photographed, using a Vickers automatic exposure unit and a Kodak Autowind 35 mm camera attached to a Leitz microscope, on Kodak AHU microfilm. Details of the developing procedure are given in Table 1.4.

Some of the specimens were further processed as follows. The tissue was dehydrated by passing it through a series of alcohols: 90%, 95%, 100%, 100%, 15 mins in each. It was cleared by immersion in creosote and stored at  $-20^{\circ}\text{C}$ . Specimens were observed and photographed as above.

#### Combining Hexaminecobaltic Chloride Dye Infusion with the GA Technique.

The dissection of the prothoracic ganglion was as described previously (Section I), with the following additional points. The pair of connectives which were not going to be at the infusion end were ligatured before the ganglion was excised from the preparation. This was done by passing

a single strand of cotton thread under each connective in turn and tying a loop as tightly as possible without breaking the connective. It was also ensured that during the excision stage the prothoracic connectives were cut as long as possible and only held at their tip. No desheathing was carried out.

The dye infusion was set up as before. The infusion was carried out for between 3.5 - 4.25 hrs. Longer times caused the specific cell fluorescence to become progressively poorer. The preparations were then developed in the ammonium sulphide. After the saline wash the ventral surface of the ganglion was desheathed and the GA procedure carried out as before (Table 1.6). Again the preparations were observed and photographed using the Leitz Dialux 20 system.

#### Intracellular Injection of Lucifer Yellow

The dissection of the thoracic ganglia was carried out as described previously (Section I), except that only the prothoracic ganglion was desheathed. The preparation was mounted on the plastic slide with the prothoracic ganglion over the araldite ridge and quickly transferred into a glass fronted perspex bath containing saline (Fig. 1.30a, b).

To keep the bath solution circulating and oxygenated, a gentle stream of oxygen was constantly bubbled through it. The slide was supported at an angle of about  $45^{\circ}$ , and viewed from the front using a X80 magnification Nikon zoom binocular microscope. The preparation was illuminated



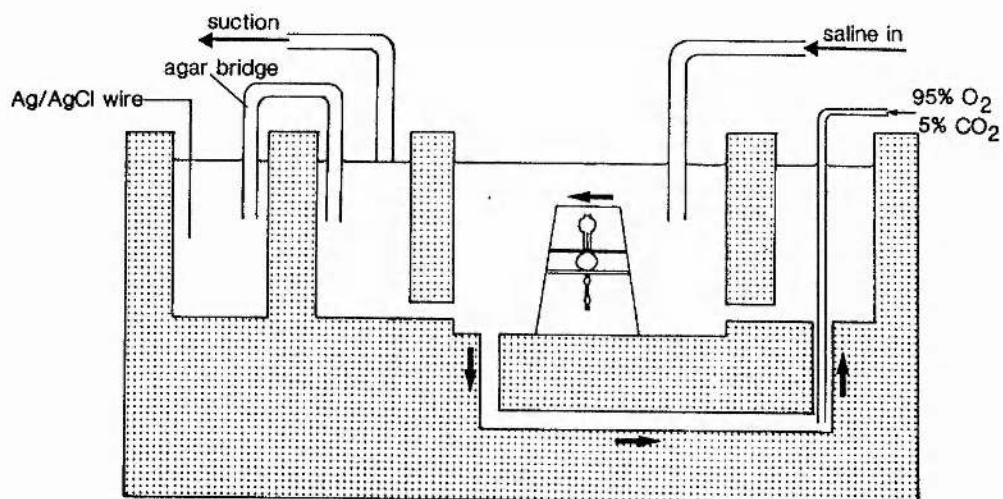
Figure 1.30 a and b The experimental bath used for intracellular recording and dye injection of neurone cell bodies. (a) represents a front view of the bath and (b) a side view.

Important features include:

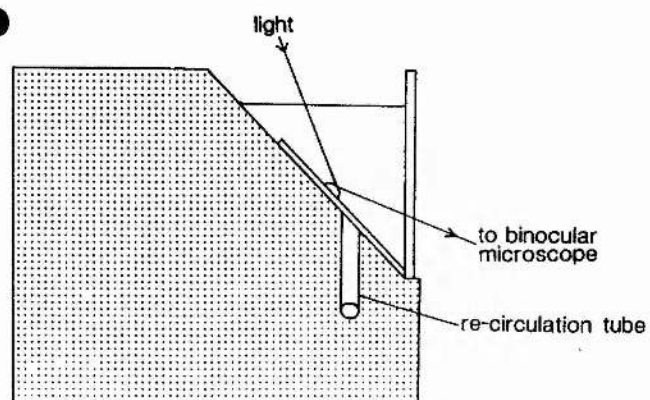
1. Preparation is mounted at an angle of  $45^{\circ}$ .
2. The  $\text{CO}_2/\text{O}_2$  gas mixture oxygenates the tissue and causes a constant recirculation of the saline in the direction shown.
3. Suction is used to maintain the saline solution at a constant level.

These diagrams are reproduced by the kind permission of Dr. K. A. Pitman.

**a**



**b**



from behind with the light passing through a movable lens and being focused onto the ganglion. By moving this lens the light path could be finely adjusted so as to illuminate any region of the ganglion. The angle of incidence could also be adjusted until the cells were seen as clearly as possible. To aid the visualization of the neurones some of the surrounding glial cells were removed by gently squirting the ganglion surface with a small jet of saline from a syringe equipped with a broken tipped microelectrode.

### Microelectrodes

The microelectrodes ( Clark Electromedical Instruments) were made from thin walled filament glass tubing with an outside diameter of 1.0 mm and an inside one of 0.78 mm. The electrodes were pulled on a Narashige type PE-2 vertical puller. The tip of the microelectrode was filled with a 3% Lucifer Yellow solution, with the rest of the shank and the electrode holder being filled with 0.1M lithium chloride. Their resistance was between 15-18 Mohms.

### Recording and Display Apparatus

Figures 1.31 and 1.32 show the set up used for microinjection and recording. The microelectrodes were held in a perspex holder which was attached to a 4mm plug fitted into the input of a unity gain high input impedance D.C. preamplifier. The head-stage of the apparatus was held vertically in a Prior micromanipulator. The output from this was fed into a differential

Figure 1.31 Block diagram of the apparatus used for intracellular recording and dye injection of neurone cell bodies.

Features to note include:

1. Remote switching between recording amplifier and constant current source is achieved using a reed relay in the probe.
2. Current is monitored by the virtual earth circuit.

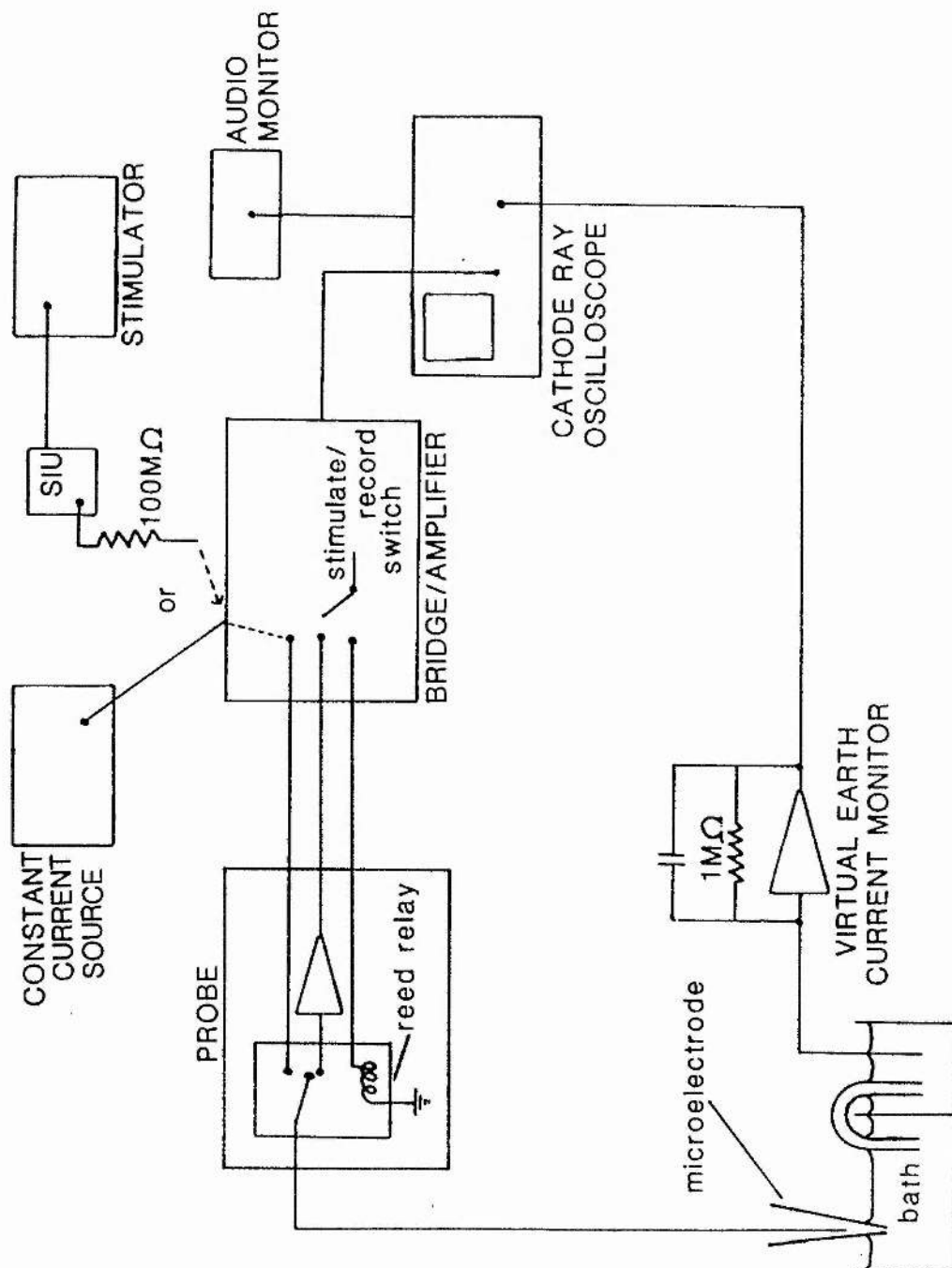
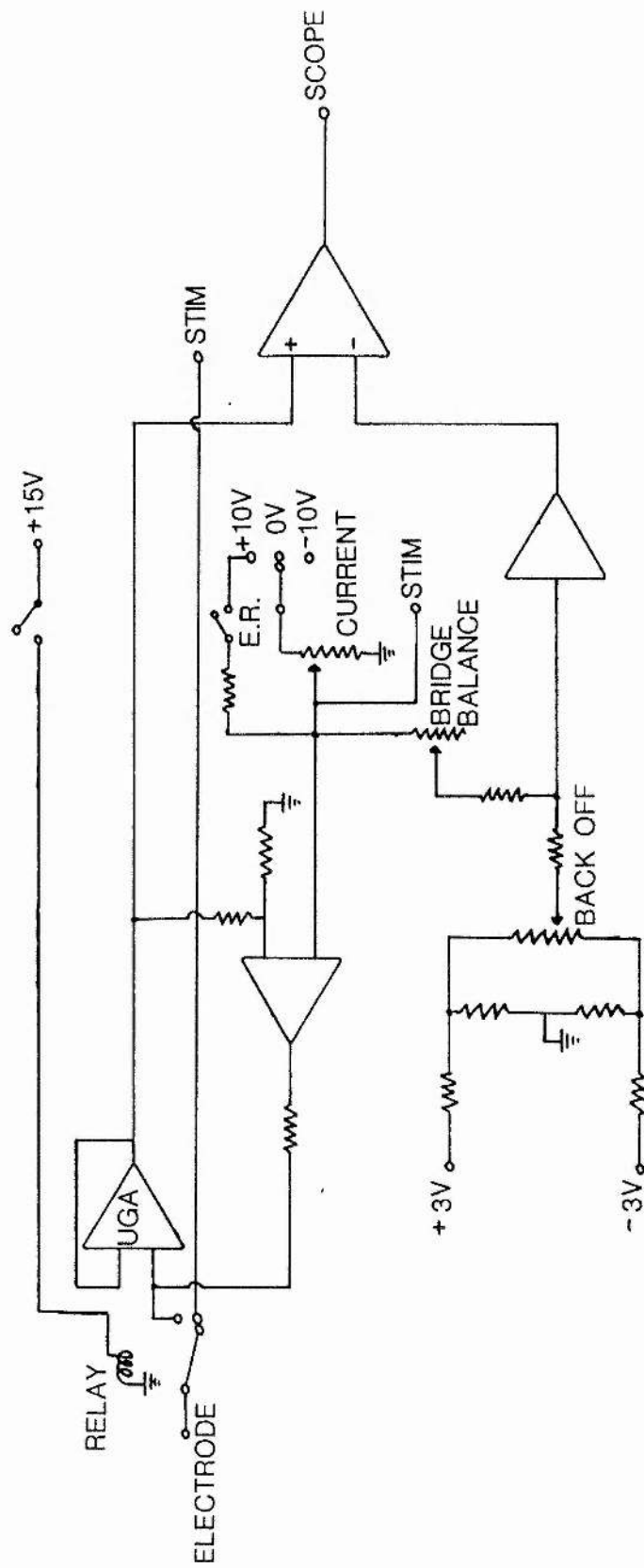


Figure 1.32 Simplified circuit diagram of the apparatus used for intracellular recording and stimulation.

Features to note include:

1. The relay and the unity gain high input impedance amplifier (UGA) were mounted inside a preamplifier probe.
2. Electrode could be connected to the stimulator (STIM) directly (as shown) or through the amplifier circuitry by energizing the relay.
3. Cells could be polarized during the course of the experiment using the bridge circuit.
4. The outputs from the back-off and the unity gain amplifier were fed into a differential amplifier and then into the oscilloscope.
5. Electrode resistance could be checked at any time during an experiment (ER).





amplifier which was equipped with a bridge circuit to allow the cell membrane to be polarized during recording. The resistance of the microelectrode could also be monitored before and during the experiment. The output from the amplifier was then fed into the oscilloscope. Current could be passed into the microelectrode either through the circuitry of the amplifier or directly from the current source. This was achieved by the means of a reed relay in the probe. Currents passing through the bath were monitored by measuring the potential difference across a 1 Mohm resistor incorporated into the 'virtual earth' circuit. To inject the Lucifer Yellow, current was passed directly into the microelectrode from a WP instruments micro-iontophoresis programmer.

#### Penetration and Injection of the Cells

The toluidine blue, cobalt infusion and GA pictures of the ganglion were used to select a possible biogenic amine-containing medial cell. Penetration and injection was carried out as follows. The microelectrode was carefully positioned over the cell body and gently lowered until it just touched the surface of the cell. A slight change in the potential recorded at the microelectrode tip occurred at this point and could be seen on the oscilloscope. Sometimes the surface of the cell could be seen to dimple. Short duration negative current pulses were applied and the microelectrode very slowly lowered until cell was penetrated. If this did not occur the microelectrode was raised, repositioned and another attempt made.

Lucifer Yellow is negatively charged and so injection was carried out by passing constant 50 nA, 0.5secs duration, hyperpolarizing pulses at a frequency of 1 Hz through the microelectrode. This was continued for 15 mins.

The preparation was then placed in saline containing 15 mM sucrose on a glass-backed aluminium slide. It was observed and photographed as described in Section I using the Leitz Dialux 20 set up. The next step was to carry out the GA procedure. The saline plus sucrose solution was sucked off the preparation which was then put through the GA procedure as described in Table 1.6. Finally, it was observed and photographed as before.

## RESULTS

### Staining with Toluidine Blue

Toluidine blue stains all the cells which lie on the surface of a ganglion and so gives an over-view of neurone position. It may be seen that on the ventral surface of the prothoracic ganglion the neurones are located in two main groups at the anterior and posterior ends of the ganglion (Fig. 1.33).

### Hexamminecobaltic Chloride Dye Infusion

In the case of the preparations in which the cobalt had been infused into one of the prothoracic anterior connectives, it was found that the medial cell pair still fluoresced, but that the contralateral pair of the two pairs of very small lateral fluorescent cells (Fig. 1.5) were no longer present. Instead a small pair of cobalt filled neurones were seen in the appropriate position (Fig. 1.34). This suggests that these cells project into the contralateral anterior connectives.

Unfortunately the results of the cobalt infusion into the posterior connectives were not as clear cut. In every preparation the two pairs of small lateral cells were seen which suggests that these cells only project into the anterior connectives (Fig. 1.35a). In 3 out of 6 of the preparations only one of the medial fluorescent pair was seen (Fig. 1.35a), but in the other 3 both cells were visible. Also, in the ganglia in which the ipsilateral fluorescent cell was absent there was usually no obvious

cobalt filled cell which could have corresponded to it. In the preparation that did show a possible cobalt filled amine-containing neurone it was only under incident light that the cell was visible (cf Fig. 1.35a and b). Under transmitted fluorescent light the combination of the dark staining and the background autofluorescence made it difficult to make out anything in the dark stained areas of the ganglion.

#### Intracellular Injection of Lucifer Yellow

An example of an adult cockroach prothoracic ganglion after GA is shown in Fig. 1.36. The photograph was taken before the ganglion had been cleared by xylene. Although most of the fluorescent neurones were obscured by tracheae (only the medial pair can be seen clearly), the large tracheae were in fact useful guides for locating the medial cells' position in untreated ganglia.

Nerve 5 of the three thoracic ganglia contains, among others, the axons of three inhibitory motoneurones; D1, D2 and D3 (Pearson and Iles, 1971; Pearson and Fourtner, 1973). The position of the D1 and D2 pairs, as demonstrated by cobalt dye infusion into both nerves 5, is shown in Fig. 1.37a. The location of the motoneurones seen following cobalt infusion into the mesothoracic nerve 2Ar2 is shown in Fig. 1.37b. Using the last two diagrams the positions of some of the larger cells were identified in prothoracic ganglia stained with toluidine blue (Fig. 1.33). The D3 pair were identified using the results of Iles (1976) and Pearson and Fourtner (1973).

From the above figures it was concluded that the fluorescent medial pair lay inside an area delimited by the three inhibitory motoneurons (D1, D2, and D3) and the larger motoneurons of mesothoracic 2Ar2 (Fig. 1.38). If a desheathed ganglion is correctly illuminated when it is in the experimental bath (Fig. 1.30) it is usually possible to identify most of these cells.

The next stage was to pick out a neurone of the correct size in the delimited area and attempt to fill it with Lucifer Yellow. It was, however, found to be very difficult to locate and fill the catecholamine-containing medial cells. Frequently one of the smaller motoneurons which sends its axon into mesothoracic nerve 2Ar2 was filled instead (Fig. 1.37b). A Lucifer Yellow filled cell photographed just after dye injection is shown in Fig. 1.39a. Nerve fibres could be seen more clearly from the dorsal surface (Fig. 1.39b), where the axon is seen descending into the contralateral posterior connective. This then leaves the CNS via mesothoracic nerve 2Ar2. The same ganglion after treatment with GA is shown in Fig. 1.39c. The Lucifer Yellow filled cell may be seen to be situated between the catecholamine-containing pair.

On comparing Figs. 1.39a and c it actually looks as if different cells have been filled with Lucifer Yellow. In Fig. 1.39a, however, the ganglion was unfixed, whilst in Fig. 1.39c it had been dried flat onto the slide. This example shows clearly that the drying procedure can distort cell position. This is due to: (i) the loss of the 3-

dimensional shape and spreading out of the ganglion as it is flattened and (ii) the fact that sometimes ganglia flatten at an angle, instead of straight down, which causes many of the neurones to be shifted to one side. These points have to be taken into account when using GA specimens to locate cells in untreated preparations and adds to the difficulty of identifying the correct cell.

Another major problem was that Lucifer Yellow filled cells frequently burst. This sometimes occurred during the dye injection itself and frequently during the subsequent GA treatment.

Figure 1.33 Ventral view of an adult cockroach prothoracic ganglion stained with toluidine blue. Calibration 100  $\mu$ m.

Figure 1.34 Prothoracic ganglion (ventral view) after cobalt backfilling into the left anterior connective, followed by treatment with glyoxylic acid. It may be seen that both members of the medial pair of cells are still visible; but that the two contralateral cells of the two pairs of the much smaller and more anterior fluorescent cells have been replaced by a pair of cobalt filled neurones (►).

Calibration 100  $\mu$ m.







Figure 1.35 a and b Prothoracic ganglion (ventral view) after cobalt back-filling into the left posterior connective, followed by glyoxylic acid.

(a) Under conditions for observation of fluorescence. Both pairs of the small lateral cells are visible (►); only the contralateral cell of the larger fluorescent medial pair may be seen. Note, however, that there is no cobalt filled neurone visible which is in the correct region to be considered as the partner of the fluorescing cell.

(b) Same ganglion viewed under ordinary transmitted light. There is now a cobalt filled cell body which could be the partner to the fluorescing cell seen above (>).

Calibration 100  $\mu$ m.

a



b

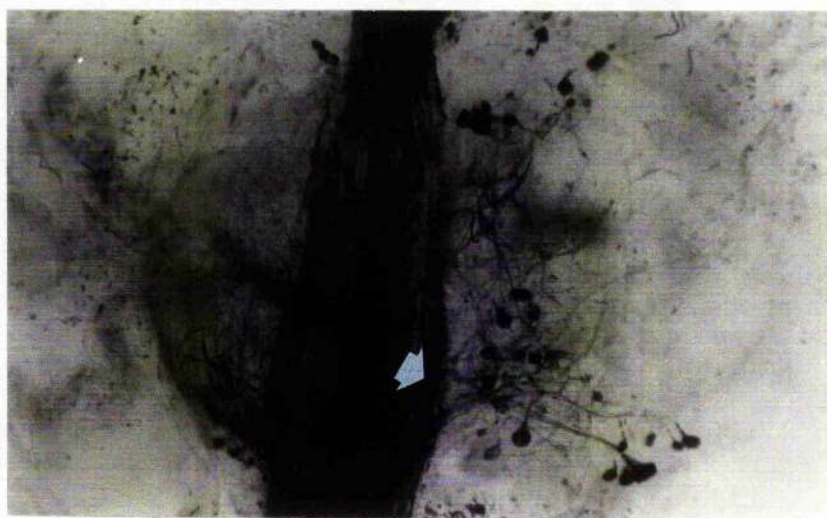


Figure 1.36 Prothoracic ganglion of an adult cockroach (ventral view) after glyoxylic acid, but prior to clearing in xylene. The only cell bodies clearly visible are the medial pair. Note that before clearing, the tracháea are quite distinct (►) and can be used to help locate the position of cell bodies. Calibration 100  $\mu$ m.

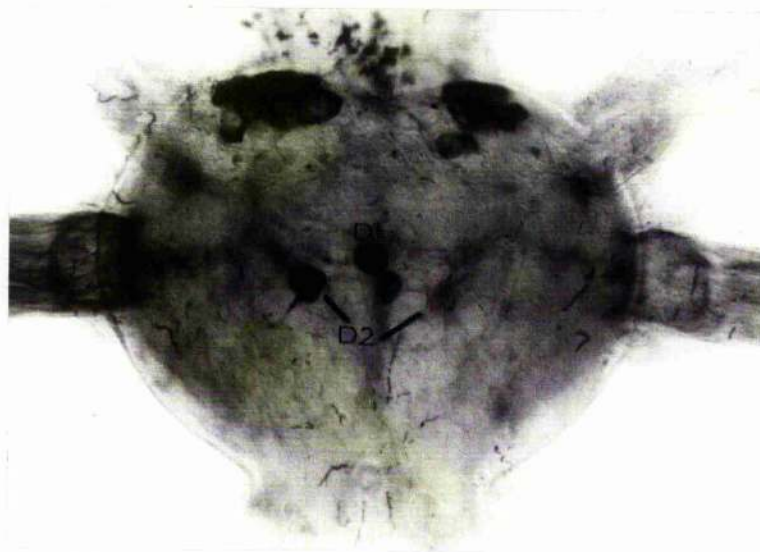


Figure 1.37 a and b Prothoracic ganglion of an adult cockroach after cobalt back-filling into two peripheral nerves. Both preparations were dehydrated and cleared.

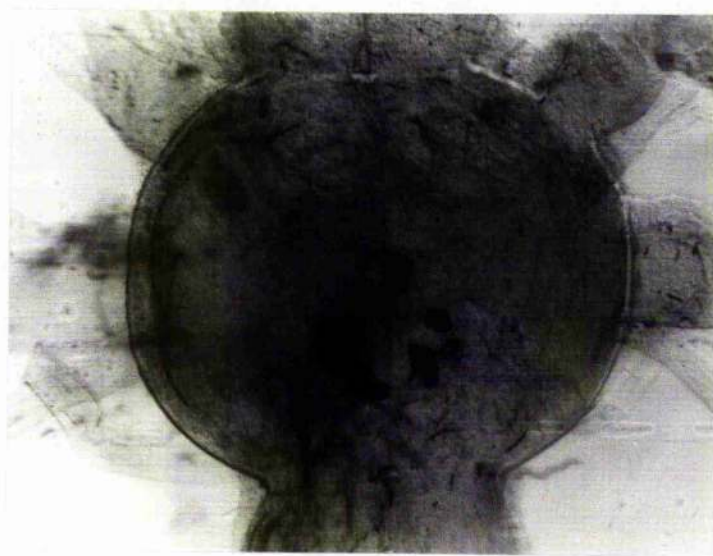
(a) Both nerves 5 have been filled. The cell bodies of two pairs of inhibitory neurones, D1 and D2, may be seen in the centre of the ganglion.

(b) The left mesothoracic nerve 2Ar2 has been filled. Several cell bodies lying both ipsi- and contralateral to the filled nerve are visible.

Calibration 200  $\mu$ m.



a



b

Figure 1.38 Diagram of Fig. 1.33 showing the location of the three inhibitory motoneurone pairs (D1, D2 and D3) and two pairs of neurones which project into mesothoracic nerve 2Ar2. The position of the fluorescent catecholamine-containing medial pair seen in Fig. 1.36 is also shown. Calibration 200  $\mu$ m.

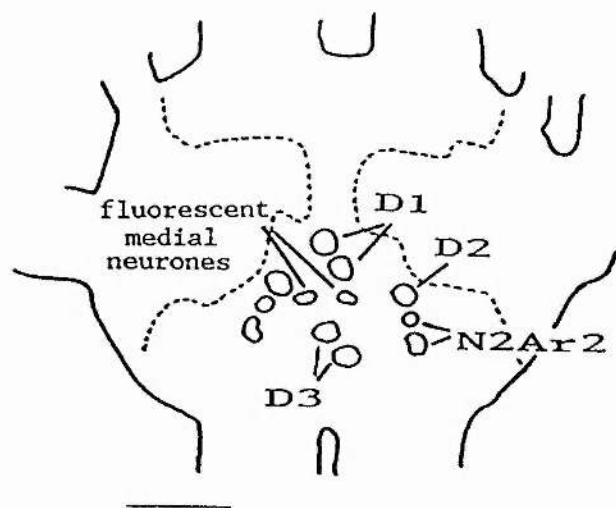




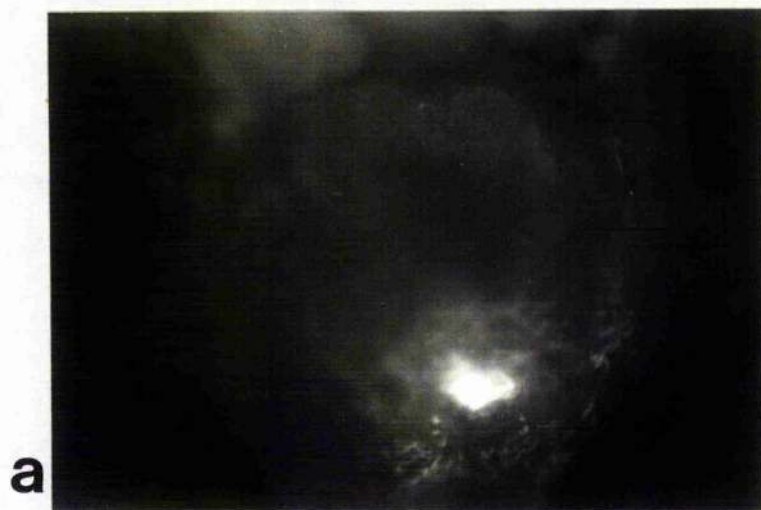
Figure 1.39 a, b and c Prothoracic ganglion of an adult cockroach after intracellular injection of Lucifer Yellow and treatment with glyoxylic acid.

(a) Ventral view showing position of Lucifer Yellow filled cell.

(b) Dorsal view showing axon descending into the contralateral posterior connective. It then left the CNS through the mesothoracic nerve 2Ar2.

(c) Ventral view showing the fluorescent medial pair plus the Lucifer Yellow filled cell body (►).

Calibration 100  $\mu$ m.



DISCUSSION

Dye infusion of hexaminecobaltic chloride was primarily carried out in order to determine the axonal projections of the medial pair of fluorescent cells. Unfortunately the results were inconclusive, although the axon projections of the two pairs of small lateral <sup>neurones</sup> were determined. With regard to the larger medial pair, the results indicate that they do not project into the anterior connectives, but with respect to the possible posterior axonal projection the results are unclear. Although for some specimens poor dye infusion could explain why both cells fluoresced, for one specimen in particular this was not the case. The other problem was that usually no cobalt filled partner was readily identifiable in those cases where only one cell did fluoresce. However, it is worth noting that it was always the cell ipsilateral to the infusion that was absent. It could be argued that this was simply due to the black staining of the surrounding tissue, but then the much smaller more anterior ipsilateral pair were clearly visible even though they overlaid densely stained tracts. With insect neurones it has been found that the segment of axon immediately connected to the cell body is thinner than that further down. Hence it is possible that not enough cobalt was present in the cell body to stain it black on development in sulphide, but that there was enough to quench the fluorescence. It is worth remembering here that it is only after silver intensification that most of the dendrites of a neurone intracellularly injected with cobalt are stained.

Hence the data shows that the two pairs of small lateral

cells project anteriorly and suggests that the axons of the medial pair may project posteriorly, although the evidence for this is still far from conclusive.

Even though preliminary experiments were carried out to aid the identification of the amine-containing medial cells, no totally successful intracellular injection of Lucifer Yellow was achieved. Ganglionic distortion during the drying procedure leading to apparent changes in neurone location was mentioned previously. More fundamental is the fact that neurone position does actually vary significantly for the same ganglion in different cockroaches; a specific neurone will be located within a small region of the ganglion surface rather than at a precise point (Pearson and Fourtner, 1973). The overall neuronal pattern does, however, remain constant (Cohen and Jacklet, 1967). Although this pair of neurones is the largest of the fluorescent cells in the prothoracic ganglion, they are in fact relatively small (about 20  $\mu\text{m}$ ) compared with many other cockroach neurones commonly used for intracellular recording (Cell 28 is about 80  $\mu\text{m}$ ; the DUM neurones about 40  $\mu\text{m}$ ). It is also possible that the cells could be among the neurones that lie slightly below the ganglion surface. This would not be detectable from the fluorescence slides but would add to the problem of identification and impalement.

The small size of the cells picked for intracellular injection could be one factor to explain cell bursting. The smaller the cell the harder it is to achieve a clean penetration and the greater is the degree of injury once it

does occur. Hence even if a small cell survives the injection it will be more likely to burst during the GA procedure, especially since the ganglion is incubated in a solution which aids GA penetration into the neurones. Thus, although one of the reasons for using Lucifer Yellow was that no development of the stain was necessary and hence specimens could be run through relatively<sup>quickly</sup>, this advantage was greatly diminished because of the problems with cell bursting.

Perseverance with these injections would no doubt have eventually led to the correct cell being filled, but it was felt that there were other areas and aspects of the aminergic innervation to be investigated and so these experiments were shelved.

SECTION III

THE AXONAL PROJECTIONS OF THE SUBOESOPHAGEAL (SOG)  
CATECHOLAMINE-CONTAINING NEURONES.

INTRODUCTION

As with the prothoracic ganglion, the GA technique brought about the fluorescence of a pair of cells in the suboesophageal ganglion (SOG) that appeared to send their axons into the posterior connectives. To confirm this cobalt dye infusion was combined with the GA technique.

The only peripheral nerves which consistently showed any fluorescent axons were the pair of salivary duct nerves (SDN) of the SOG. This nerve, along with the stomatogastric nerve of the stomadeal nervous system constitute the only innervation of each salivary gland (Whitehead, 1971). The SDN runs down the main salivary duct, crosses to the reservoir duct and then branches extensively to innervate acini, ducts and muscle fibres in the reservoir wall.

There is conclusive evidence for a functional role for the SDN (House and Smith, 1978; Ginsborg and House, 1976), whereas that for the stomatogastric nerve is still incomplete. There are also several lines of evidence that support the idea that dopamine is the transmitter at the neuroglandular junction of the SDN. For example, methylene blue staining (Whitehead, 1971; Bowser-Riley, 1978) and scanning electron microscopy (Bowser-Riley, 1978) have shown that the gland acini have a profuse plexus of nerve fibres containing numerous varicosities of a similar size to those demonstrated by the Falck-Hillarp technique (Bland et al., 1973). This provides evidence that these varicosities contain a catecholamine. The stimulation of the SDN terminals causes the release of dopamine (Kapoor, Verma -



Kapoor, Whale and Arbuthnott, 1983). Exogenously applied dopamime mimics nerve stimulation by producing electrical changes in the acinar cells (Ginsborg, House and Silinsky, 1974) and fluid secretion by the entire gland (House and Smith, 1978). Evidence also exists for dopaminergic innervation of salivary glands in other insects (Klemm, 1972; Robertson, 1975).

To determine if any of the fluorescent cells in the SOG project into the SDNs, these nerves were backfilled with hexaminecobaltic chloride. If the neurones shown up by this correspond to positions of fluorescing neurones then this would confirm that the SDN contains efferent dopaminergic fibres with their cell bodies in the SOG.

From the GA experiments it was found that all the fluorescent neurones are paired, except one; it is located on the midline of the SOG. From experiments in which the SOG had been cut horizontally before the GA incubation it was found that this neurone is present on the dorsal surface. The only other unpaired neurones which have been studied to any degree are the DUM neurones. These are located along the midline of the dorsal surface of each ganglion and are thought to be octopaminergic (see Evans, 1980, for a review). This cell is, therefore, rather unique amongst the catecholamine-containing neurones. It is also a reasonable size (about 15-20  $\mu\text{m}$ ) and so it was decided that it would be worth concentrating on this neurone, with the ultimate aim being to characterize its morphology and function by intracellular techniques. As noted in the results (pg 74)



Klemm (1971) has also observed some paired dorsal medially located dopamine-containing neurones in the thoracic and abdominal ganglia of Trichoptera. It is not known if these neurones are in any way homologous to the one described here.

The earlier experiments (Section I) have shown that the cell has a bifurcating axon but it was impossible to follow it in the neuropile for any distance. Previous experiments (Section II) have also shown that Lucifer Yellow injected cells tend to burst, especially during the GA incubation step. Hence it was decided to try both hexaminecobaltic chloride and horseradish peroxidase intracellular injection instead. Also, in insect preparations Lucifer Yellow injection does not give as much information about cell morphology as the other two. This is in part due to interference caused by background fluorescence of the tissue.

It has been shown above (Section II) that the GA technique can be used along with cobalt back-filling and so it was thought that it would also work with intracellularly injected cobalt. The main drawback with this is that the correct cell is identified by the fact that it is stained black and does not show up in the subsequent GA incubation. But if the GA incubation did not work well then the same result would be seen, even though the cell that was filled was the wrong one.

The combination of GA and horseradish peroxidase (HRP)

would, in theory, give much clearer results. HRP staining has been used as a neuroanatomical tool in both vertebrates (Lavail and Lavail, 1972) and invertebrates (Muller and McMahan, 1976; Watson and Burrows, 1981). In 1975 Ljungdahl, Hokfelt, Golstein and Park combined retrograde peroxidase tracing with immunohistochemistry in their investigation of rat brain dopaminergic neurones. Then a few years later Berger, Nguyen-Legros and Thierry (1978a and b) and Berger and Nguyen-Legros (1979), also working on rat mesencephalon, combined HRP retrograde staining with GA-induced fluorescence. In both cases the HRP was injected first and 24 hours later the rats were killed, and their brains fixed and sectioned. Next, the sections were processed by immunocytochemistry or GA; and finally the neurone was visualized using the HRP. The advantage of this is that the HRP does not interfere with the fluorescence and so it is possible to observe the same neurone shown up first with fluorescence and subsequently with the oxidised chromogen.

## MATERIALS AND METHODS

### Toluidine Blue Staining of the Suboesophageal Ganglion

This was carried out as described for the prothoracic ganglion (Section II). It was used to show the position of the neurones and their size distribution in the ganglion.

### Combination of Hexaminecobaltic Chloride Dye Infusion with the GA Technique

The dissection of the suboesophageal ganglion (SOG) was as described in Section I. The procedures for setting up the dye infusion, combining the two techniques and photographing the results were the same as those used for the prothoracic ganglion (Section II).

### Hexaminecobaltic Chloride Dye Infusion into the Salivary Duct Nerve

The salivary duct nerves (SDN) are paired and exit from the SOG at its posteroventral margins. They are very fine nerves and are usually enveloped by fat body. Several small labial nerves also emerge at this point. Methylene blue staining has shown that the SDNs are composed of at least two axons (Whitehead, 1971). Cross sections of the salivary gland show that the branches of the nerve contain two large axons 7  $\mu$ m in diameter, and several smaller ones (Whitehead, 1971). After leaving the ganglion each SDN becomes closely associated with small tracheae and both of these pass posteriorly in a channel formed between the connectives and the ventral cervical muscles. Once the nerves have reached the dorsal end of the second ventral sclerite they leave the

tracheae, cross over to the salivary duct and follow the lateral margins of this duct down to the salivary glands.

The dissection of the SOG was carried out as described in Section I as far as the removal of the maxilla. The next step was to cut the salivary duct as far anterior as possible, grasp it with forceps, and slowly and carefully draw it away from the preparation until the SDNs were seen to join the duct. Then as the duct was being pulled away the nerves were gently separated from it. At the point when the duct bifurcates the nerves were cut. Each nerve, held only at its tip, was carefully pulled back and the tracheae which run from it to the cervical muscles cut. The SOG was then excised as described before, placed in some saline and the adhering connective tissue, tracheae and fat body removed, especially from around the SDNs. No ganglionic desheathing was carried out.

The cobalt infusion was set up as described in Section II, great care being taken to handle the SDN as little as possible since it could be very easily damaged. The preparation was left for 36 hours before the cobalt sulphide was precipitated out. The preparations were taken to 70% ethanol in saline solution as described in Section II. They were then silver intensified (see below).

#### Wholemout Silver Intensification of Cobalt-Filled Neurones

A number of methods have been developed for the wholemount intensification of cells (Strausfield and Obermayer, 1976; Bacon and Altman, 1977). The method used in these

experiments differs from these in some respects and incorporates a step based on the destaining procedure of Pitman (1979b). Table 1.8 gives the protocol for the intensification procedure. The preparations are put through this straight from the 70% ethanol in saline solution. The destaining steps were introduced to remove any contaminants from the surface of the ganglion. This was especially necessary when intracellular injection of the cobalt salt was carried out (see later experiment), where silver from the electrodes was one of the contaminants. These steps greatly reduced the non-specific darkening of the ganglion during the intensification and so improved the contrast between the stained cells and the surrounding tissue.

Whilst in the silver solution the preparations were briefly observed with minimum light under a binocular microscope every 10 min and then returned to the dark. Gradually the axon and dendrites of the filled cells became visible and turned progressively blacker. At this stage preparations were observed more frequently. The optimum state was reached just at the point when the ganglion surface also started to darken. As soon as this was seen to be happening the intensification was stopped by transferring the preparation to solution (9). The citric acid in this solution lowers the pH from about 5 to 2-2.5. Below pH 2.3 the intensification proceeds very slowly and so the reaction is quickly stopped.

After step (13), preparations were dehydrated in an ascending series of ethanols (20 min in each of the following: 70%, 90%, 95%, 100%, 100%). Finally they were cleared in creosote and stored in this solution at  $-20^{\circ}$  C.

TABLE 1.8

SOLUTION	TEMP (°C)	TIME
*1. 1% Potassium fericyanide + 1.25% sodium thiosulphate in 50% ethanol (potassium ferricyanide dissolved in the sodium thiosulphate solution immediately before use).	Room Temp.	5 min.
*2. Wash in 1.25% sodium thiosulphate in 50% ethanol.	Room Temp.	10 min.
3. 50% ethanol.	Room Temp.	10min.
4. 1.25% gum acacia in 30% ethanol.	45°	15 min.
5. 2.5% gum acacia in 30% ethanol.	45°	15 min.
6. 5% gum acacia in 30% ethanol.	45°	15 min.
7. 1.6% citric acid + 2% hydroquinone in solution (6).	45°	1 hour.
8. 0.1% silver nitrate in solution (7) ( <u>in the dark</u> ).	Room Temp.	0.5-1.5 hours.
9. 1.6% citric acid in solution (6).	4°	10 min.
10. 1.6% citric acid in solution (6).	Room Temp.	10 min.
11. 2.5% gum acacia in 30% ethanol	45°	15 min.
12. 30% ethanol	45°	15min.
13. 50% ethanol	Room Temp.	15 min.

Note. Metal forceps must not be used to transfer ganglia at stages marked with a \*.

Preparations can be stored under these conditions for many months before any discernable deterioration of the ganglion occurs. If stored at room temperature or  $4^{\circ}\text{C}$ , a general browning of the preparation is seen within a few days.

#### Stereoscopically Paired Photographs

The intensified preparations were mounted at the desired angle in creosote in a glass backed aluminium slide. They were observed using a Leitz microscope fitted with a Zeiss 'Luminar' lens. The stage of the microscope could be tilted to two different positions and this allowed preparations to be viewed and photographed from two angles which differed from each other by  $15^{\circ}$ . The photographs were taken on Kodak AHU microfilm (see Table 1.4 for developing procedure); using a Vickers J 35 Automatic Exposure Unit and a Kodak Autowind 35 mm camera. When the paired photographs are placed side by side such that equivalent points on the two photographs are about 8 cm apart (interpupillary distance) and viewed through stereoscopic spectacles, the two images may be visually superimposed. This provides a good picture of the three dimensional shape of the neurone.

#### Intracellular Injection of Hexaminecobaltic Chloride Combined with GA Fluorescence

The set up used for the injection was similar to that described for the injection of Lucifer Yellow (Section II). The freshly dissected SOG (see Section I) were desheathed on their dorsal surface before being placed in the perspex bath. A big problem with this preparation was that it was very



difficult to make out the cells on the dorsal surface. The circumoesophageal connectives invariably got in the way. Also, some of the dorsal neurones lie in a funnel shaped group of cells (see results below) which extends through the ganglion. It is very difficult to impale cells that do not lie on the surface.

In these experiments the microelectrodes were filled with a solution of 100 mM hexamminecobaltic chloride. The holder was filled with 2 M potassium acetate. Their resistance was usually 10-20 Mohms. To inject the dye, 0.1  $\mu$ A positive current pulses of 0.5 sec duration and frequency of 1 Hz were passed through the microelectrode. This was continued for 20 min and then the electrode was withdrawn and the preparation placed in a vial containing about 5 ml. of saline and one small drop of ammonium sulphide solution. After the cobalt sulphide was precipitated out and the preparation washed, the ventral surface of the ganglion was desheathed and the GA procedure carried out (Table 6). The preparations were then observed and photographed using the Leitz Dialux 20 system.

#### Intracellular Injection Of Horseradish Peroxidase Combined with GA

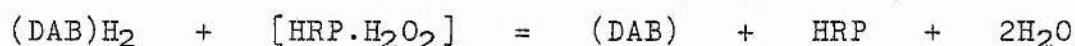
The apparatus was the same as that used for the other intracellular injection experiments.

The microelectrodes were filled with a solution of 4% HRP (Sigma, Type VI) in 0.2 M Tris buffer (pH 7.4) with potassium chloride added to 0.5 M. Their resistance was 10-20 Mohms. The HRP was injected by passing 5 nA of positive



current in 500 ms pulses every second for 30-60 min. The preparation was then removed from the bath and incubated in saline for 1-2 hr to allow for further diffusion of the dye into the processes of the filled cell. The ventral surface of the ganglion was then desheathed and the GA procedure carried out as described in Table 6, except that the preparations were mounted in mineral oil.

After HRP has been injected into a cell it is incubated in a medium containing hydrogen peroxide ( $H_2O_2$ ) and 3-3' diaminobenzidine tetrahydrochloride (DAB; Sigma) which assumes a dense brown colour only in the oxidised state. At sites containing HRP, oxidation takes place and the DAB becomes visible. The reaction is as follows:



Since DAB may be carcinogenic, rubber gloves were worn and the reaction carried out under a ventilation hood.

Once the preparations had been photographed to record the positions of the fluorescent cells, the mineral oil was washed off using xylene and the DAB reaction carried out on glass backed aluminium slides as follows. The tissue was presoaked for 20 min in a solution containing 0.05 M phosphate buffer at pH 7.4 and 0.05% DAB. This solution was then sucked off and the reaction initiated by adding a phosphate buffer solution which contained both 0.05% DAB and 0.01% hydrogen peroxide. The preparation was frequently observed under a binocular microscope. Once the optimum intensity was reached the reaction was stopped by rinsing the preparation with saline. The ganglion was dehydrated by

transferring it rapidly through an ascending series of alcohols (5 min in each), and then cleared and mounted in creosote. The Leitz Dialux 20 system was again used for photographing the preparations.

## RESULTS

### Toluidine Blue Staining

In both the locust and the cockroach the interganglionic connectives of the thoracic ganglia emerge from the anterior and posterior ends of each ganglion and the fibre tracts they contain run through the middle, forming part of the neuropile region. The ganglia themselves are relatively flat, especially on the dorsal surface, and the peripheral nerves emerge from the sides (Fig. 1.5; Gregory, 1974; Tyrer and Gregory, 1982). The same is found with the locust suboesophageal ganglion (SOG) (Tyrer and Gregory, 1982). In the cockroach, however, the framework of the SOG is quite different. The dorsal surface is highly curved; the circumoesophageal connectives project from the middle of the dorsal surface; and the main peripheral nerves emerge from the most anterior edge of the ganglion (Fig. 1.19; also Appendix; Willey, 1961).

After toluidine blue staining it is seen there are two main groups of neurones on the dorsal surface of the SOG: one lying between and posterior to the circumoesophageal connectives; the other lying anterior to them (Fig. 1.40). After studying the photographs of the fluorescent preparations it was decided that the fluorescent unpaired dorsal neurone lies amongst the anterior group of cells. The group of neurones lying between and below the connectives are probably homologous to the DUM neurones found on the dorsal surface of the thoracic ganglia. They are similar in size, number and distribution to the other groups of DUM

neurones and were also found to have the characteristic bifurcating axon (see results). These would, therefore, be expected to be octopaminergic. The toluidine blue specimens also showed that the anterior group form a funnel through the ganglion (Fig. 1.41). Nothing like this is found in the thoracic ganglia. Willey (1961) identified these cells as labral neurones. A group of fibres originates from each side of the cells, passes into the ipsilateral circumoesophageal connective and then out into the labral nerve (See Appendix; Willey, 1961). As mentioned previously this nerve shares a common root with the frontal connective. After GA it was not possible, in the preparations obtained, to visibly identify the labral nerve as distinct from the frontal connective. Since it has been shown that after GA the frontal connectives contain fluorescent axons (Fig. 1.26; Klemm, 1983), it was, therefore, impossible to determine whether or not the labral nerve contained the axon of the fluorescent dorsal cell.

Combined Cobalt Dye Infusion/ Glyoxylic Acid on the Suboesophageal Ganglion

For each specimen it was found that the larger of the posterior lateral pair of fluorescent neurones disappeared on the ipsilateral side when cobalt was infused into one of the posterior connectives (Fig. 1.42a and b). In neither case is it obvious which cobalt cell is the amine-containing one since there are several cobalt filled cell bodies in the correct area. It is also seen that the fluorescent cells are not always in the same position on the ganglion surface. This is mainly due to the various procedures that the

ganglia are processed through, and unfortunately means that the amine-containing cells cannot be accurately pinpointed from the cobalt infused preparations.

The results confirm that the larger posterior ventrolateral pair of neurones are not local interneurons (since their axon extends into the posterior connective), but it is not known how far they extend; cobalt infusion into connectives further down the ventral nerve cord takes too long to reach the SOG and still allow the fluorescence technique to work. During the period of cobalt infusion the concentration of the catecholamines in the neurones slowly decreases. This may be due to diffusion into the surrounding tissue and/or cessation of catecholamine synthesis in combination with axonal transport from the cell body. Hence as time progresses the level of specific fluorescence decreases until all that is left is a general background autofluorescence. The infusions in these experiments were carried out for no longer than 4.25 hours. For cobalt to reach as far as the SOG from the posterior prothoracic connectives at least 12 hours of infusion would be required, by this time the specific fluorescence developed after GA would be negligible.

#### Dye Infusion into the Salivary Duct Nerve

Silver intensification of the cobalt infused specimens showed that each salivary duct nerve (SDN) contains the axons of two cells located in the SOG. Both are on the ventral surface of the ganglion (Fig. 1.43a). The more posterior cell is ipsilateral to its SDN; it branches mainly

into the neuropile which lies contralateral to the cell body (Fig. 1.43a, b and c). The more anterior cell lies contralateral to its SDN; it branches less frequently than the other cell, mainly into the neuropile which is ipsilateral to it (Fig. 1.43c). It also projects into the circumoesophageal connective which lies ipsilateral to it (Fig. 1.44a). Axon branches from both cells appear to intermingle (Figs. 1.43b and c), especially on the dorsal surface of the ganglion, between the circumoesophageal connectives (Fig. 1.44b).

Comparison of cobalt-backfilled preparations with GA fluorescence preparations shows that there are two pairs of fluorescent cells whose positions correspond to the cobalt stained ones (cf Figs. 1.43a and 1.45). The anterior half of a SOG is shown in Fig. 1.46. The axons of the large fluorescing (and SDN projecting) neurones may be seen to extend towards the centre of the ganglion. This is what is seen with the axon of the more anterior cobalt filled neurone of the SDN (Fig. 1.43b and 1.44a). Fluorescent varicose fibres were also seen in the area between the circumoesophageal connectives (Fig. 1.20), although it is unknown from which neurones they emerged. In addition, many of the finer branches from both cells are seen to be varicose which suggests that they may be biogenic amine-containing (see Figs. 1.43 and 1.44), but is certainly not conclusive since other neurone types do have varicose processes.

These results, therefore, suggest that like the locust (Altman and Kien, 1979), the cockroach SOG contains at least

two cells that project into each SDN; and that for the cockroach these are catecholamine (probably dopamine) containing. The best way to confirm this would be to follow the dye infusion with GA. Unfortunately it is necessary to infuse the dye for 36 hours, by which time it is impossible to obtain any specific tissue fluorescence.

### Intracellular Injection Experiments

Although both types of intracellular injection were carried out several times, the fluorescent dorsal unpaired neurone was not filled. A GA treated ganglion after HRP injection is shown in Fig. 1.47a. The cell stained up after development of the HRP (Fig. 1.47b) belongs to the more posterior group of DUM neurones. This type of cell was also the one filled in the example showing cobalt injection (Fig. 1.48); the characteristic DUM neurone morphology is more clearly seen than before.

One big problem with these injections was the circumoesophageal connectives. When present they tended to mask the dorsal cells, and movement of them caused the relative positions of the dorsal surface neurones to alter. It was difficult, however, to cut these connectives close to the ganglion surface without disrupting the dorsal neurones, and one objective of the experiments was to ascertain if the dorsal neurone did in fact project into them. It is also possible that the fluorescent neurone could be one of the cells that lie below the surface in the funnel of neurones. This would certainly make it very difficult to visually locate and impale.



Figure 1.40 Diagram of the dorsal surface of a SOG to show the position of the neurone cell bodies shown up with toluidine blue. The ganglion appears elongated since the dorsal surface is very curved (see for example, Fig. 1.43c) and was flattened out for the diagram. ADN, anterior group of neurone cell bodies; DUM, dorsal unpaired median neurone cell bodies; COC, circumoesophageal connectives; PC, posterior connectives.]

Calibration 100  $\mu$ m.

Figure 1.41 A SOG after staining with toluidine blue. Anterior lateral half of the ganglion is shown. A funnel of neurone cell bodies (►) may be seen to pass right through the core of the ganglion, from the ventral (V) to the dorsal (D) surface. The dark areas on the ventral surface contain neurone cell bodies (\*). C, circumoesophageal connective.

Calibration 100  $\mu$ m.



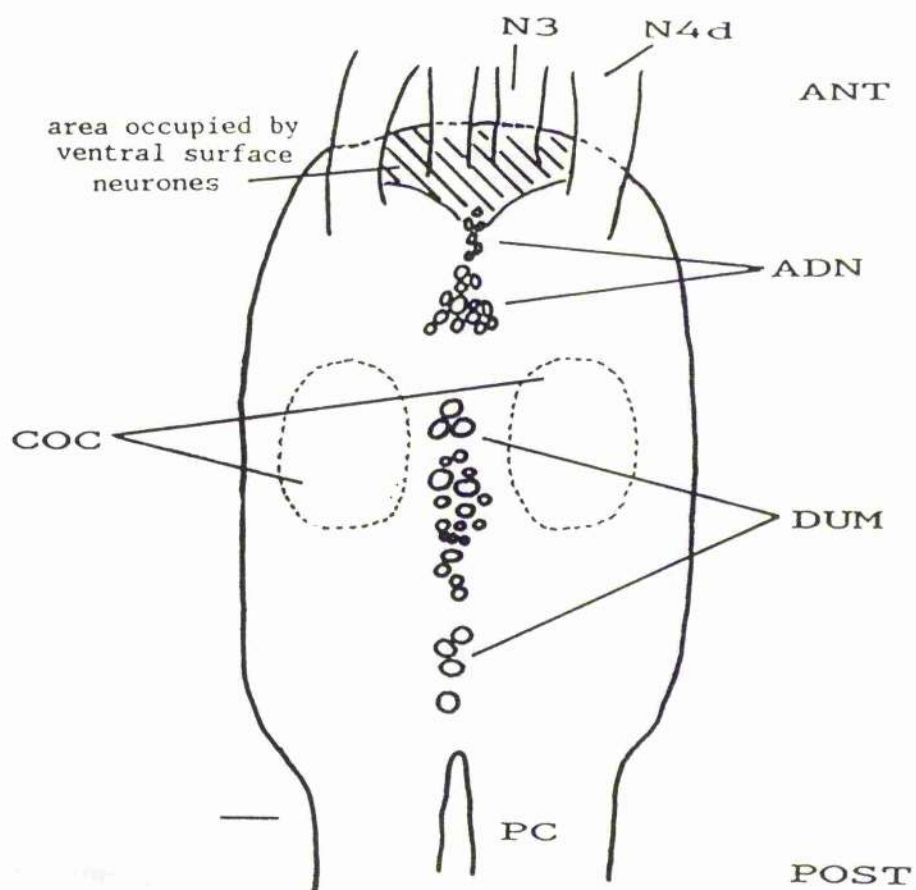


Figure 1.42 a and b Suboesophageal ganglion (ventral view) after cobalt back-filling into one posterior connective followed by treatment with glyoxylic acid. In both cases the posterior half of the ganglion is shown. It may be seen that the larger cell body of the pair of fluorescent lateral cells is knocked out on the ipsilateral side after the back-filling. It was not possible to pin-point the correct cobalt-filled cell since there were several stained cells in the correct area, especially in preparations in which good back-filling had been achieved (b). (\*), indicates the area in which the partner of the fluorescent cell is located. Calibration 100  $\mu$ m.

**a**



**b**



Figure 1.43 a, b and c Stereoscopically paired photographs of a SOG after cobalt back-filling into the left salivary gland nerve.

(a) Ventral view. Two cells are revealed; (\*). (►) marks the exit point of the nerve from the ganglion. It may be seen that one neurone lies ipsi- and the other contralateral to the filled nerve.

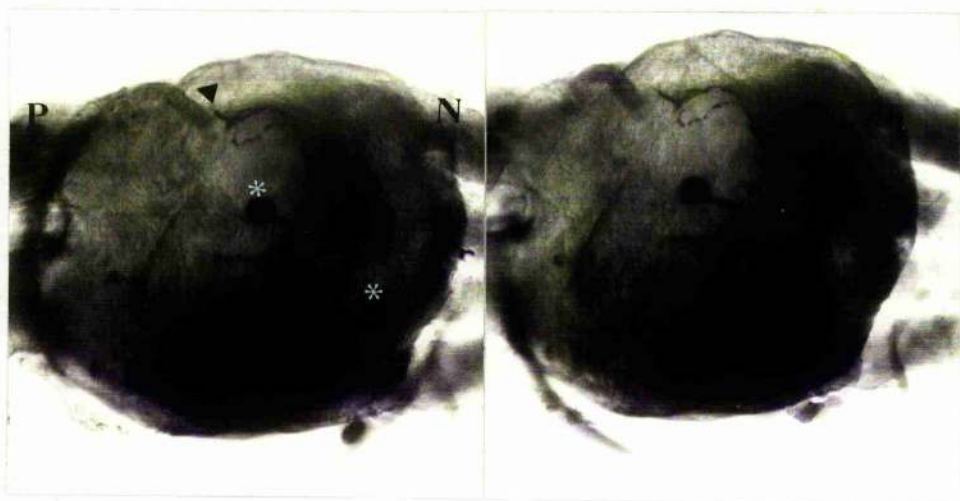
(b) Right lateral view. The nerve fibre branching of the more posterior cell (\*) may be seen to extend into the contralateral half of the ganglion.

(c) Right lateral view. The axon of the more anterior cell (\*) is seen to extend to the dorsal surface near the circumoesophageal connectives; it then branches and innervates the ipsilateral side of the ganglion as well as projecting towards the contralateral SDN.

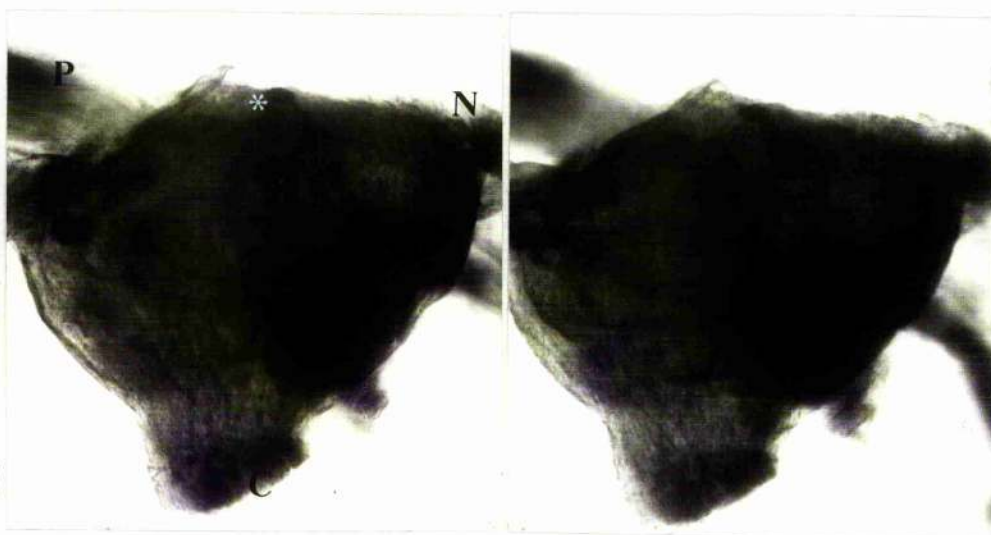
C, circumoesophageal connectives; N, nerve 4; P, posterior connectives.

Calibration 100  $\mu$ m.

a



b



c

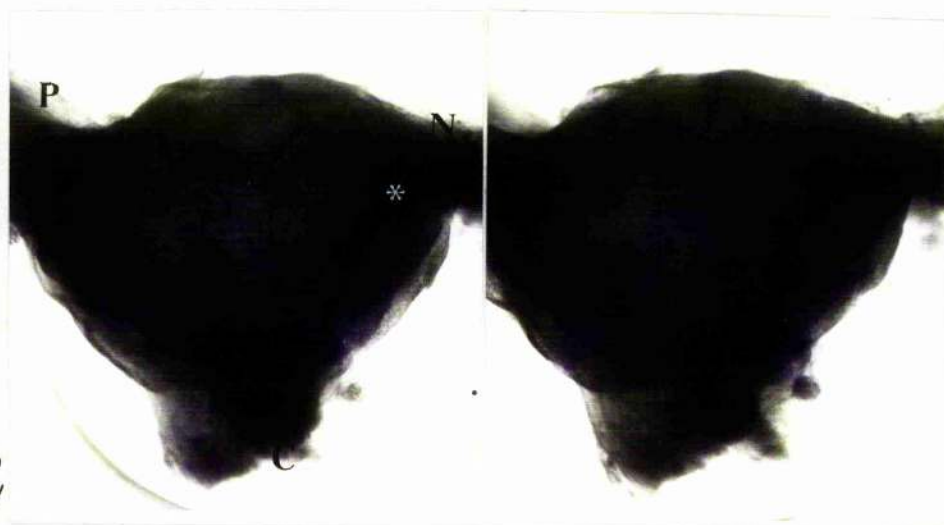


Figure 1.44 a and b SOG after cobalt back-filling into the left SDN.

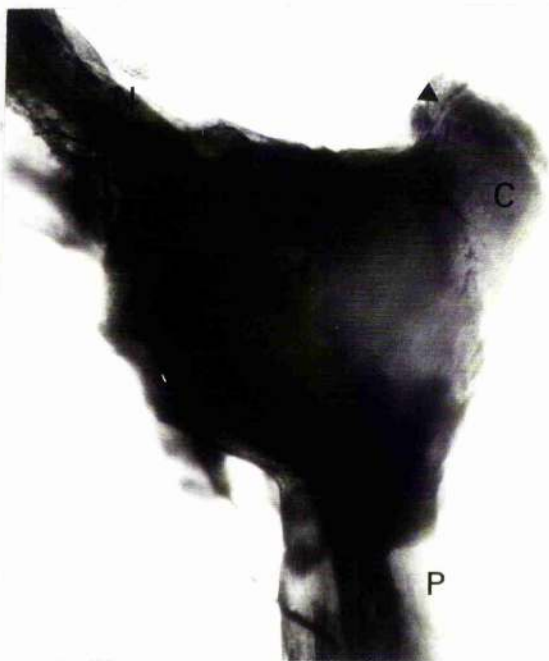
(a) It may be seen that the anterior filled cell projects into the ipsilateral circumoesophageal connective (►).

(b) The dorsal surface between the circumoesophageal connectives is shown. Branches from both filled neurones are located in this region.

C, circumoesophageal connectives; N, nerve 4; P, posterior connectives.

Calibration 100  $\mu$ m.





a



b





Figure 1.45 Same diagram as shown in Fig. 1.19 except the two pairs of fluorescent neurone cell bodies that have a similar position to those revealed by cobalt back-filling through the salivary gland nerve are indicated (►). Calibration 100  $\mu$ m.

Figure 1.46 Ventral view of a SOG after glyoxylic acid. The anterior half of the ganglion is shown. It may be seen that the axons (\*) of the large fluorescent anterior neurones (►) project towards the centre of the ganglion. Calibration 100  $\mu$ m.

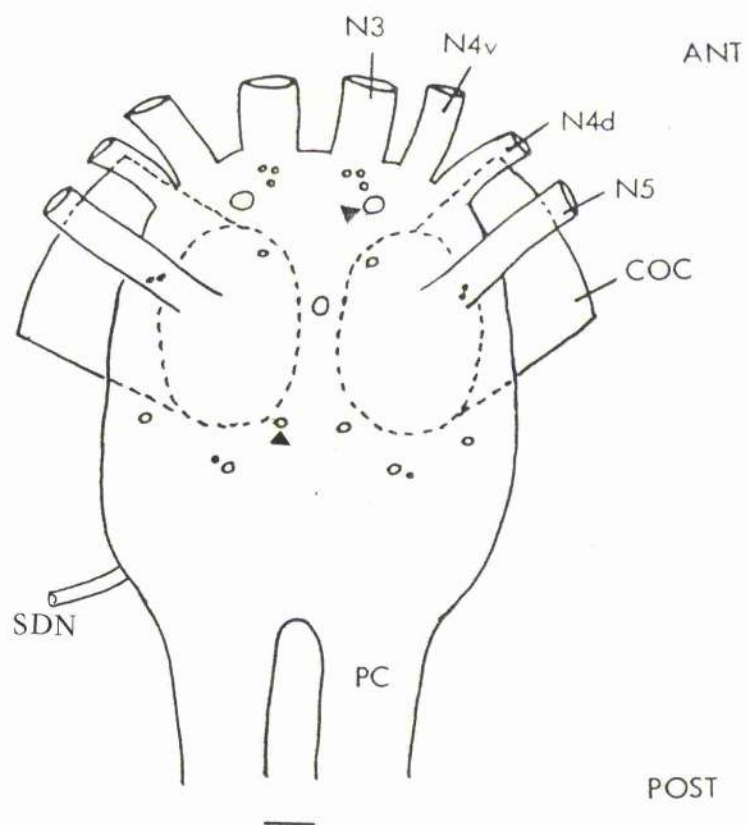


Figure 1.47 a and b Dorsal view of a SOG after intracellular injection of HRP and treatment with glyoxylic acid.

(a) Before the development of the HRP. Although most of the fluorescent neurones were not visible (since the ganglion was being viewed from the dorsal surface) it may be seen that the dorsal unpaired neurone is still present (►).

(b) The location of the HRP has been visualized with diaminobenzidine (▷). The neurone stained up is one of the DUM neurones. (O) indicates the position of the fluorescent unpaired neurone.

Calibration 100  $\mu$ m.

a



—

b



Figure 1.48 A SOG after intracellular injection of hexamminecobaltic chloride and treatment with glyoxylic acid. It may be seen that the cobalt filled cell is one of the DUM neurones - (►) shows the position of the symmetrical branches of it's axon. Lying more anterior, the fluorescent dorsal unpaired neurone is still visible (\*). A fluorescent axon (▷) is seen to run between the circumoesophageal connectives, C. It is unknown whether or not this is contained within the second substomodeal connective. However, it does appear to run between the fluorescent regions of the tritocerebrum (T), and it is known that the descending axons from these regions almost entirely cross over to the other side through the two substomodeal commissures. The fluorescent fibre containing frontal connectives (F) are also visible.

Calibration 100  $\mu$ m.



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DISCUSSION

The suboesophageal ganglion (SOG) is made up of three fused neuromeres. from anterior to posterior the mandibular, maxillary and labial neuromeres (see Appendix; Tyrer and Gregory, 1982; Willey, 1961). From the positions of the salivary duct neurones' branches in the neuropile of the SOG of the locust, Altman and Kien (1979) concluded that these neurones probably receive inputs from all the mouthparts. and that these provide a means of regulating salivary production.

The results from this study indicate that in the cockroach the salivary gland neurones are catecholamine-containing and branch into the the maxillary and labial neuromeres (see Appendix) and also right across the neuropile (from ventral to dorsal) to innervate the region between the circumoesophageal connectives. This part of the ganglion is packed with ascending and descending fibres (see Appendix) and hence it is likely that input from the cerebral ganglia also has a role in the control of salivation. One of the branches of the more anterior salivary neurone projects into the circumoesophageal connective but its destination is unknown. One possibility is that it could contact fibres from labral neurones in the tritocerebrum which send their axons out into the labral nerve.

Sectioning ganglia before silver intensification would help to clarify both the extent of salivary neurone branching in the neuropile and also the areas of overlap between the neurones themselves.



The staining with the toluidine blue revealed the funnel of cells which pass through the neuropile of the SOG. It was mentioned that nothing like this is found in the cockroach thoracic ganglia, but also there is nothing similar in the locust SOG which is composed of the same three neuromeres (Tyrer and Gregory, 1982). As with the locust metathoracic ganglion the neuromeres that make up the locust SOG appear to have fused by the loss of the pairs of connectives which initially ran between them (and for some part of the internal structure of the neuromere has been lost too). In the cockroach it is possible that the neuromeres fused in a less direct fashion with the funnel of neurones being originally located on the on the outer surface of one of these.

It is proposed that the neurones located along the midline of the dorsal surface between the two pairs of connectives are homologous to the thoracic DUM neurones. In support of this it was found that although they did not fluoresce when treated with glyoxylic acid they did stain up specifically with the dye neutral red which is part of the initial evidence used by Dymond and Evans (1978) to show that the thoracic DUM neurones were octopaminergic. To confirm this it would be necessary to microdissect out the neurones and show the presence of octopamine by biochemical means. In addition, confirmation of whether or not the axon endings were of the blind neurosecretory type would finalize the degree of homology.

Although the correct anterior dorsal unpaired neurone was

not identified it was shown that it is possible to combine intracellular injection of either cobalt or HRP with GA. This is important since when GA was combined with cobalt backfilling into the posterior suboesophageal connectives, it was found impossible distinguish the catecholamine-containing, cobalt filled neurone from the numerous other cobalt filled neurones that were near it. Hence intracellular dye injection studies would be necessary to identify this neurone (although its small size would make it difficult) and also to give more detailed information about the morphology of the two catecholamine-containing salivary duct neurones.

To overcome the problem of identifying the anterior dorsal neurone it might be helpful to attach the ganglion to a slide which had a rubbery rather than a hard araldite bridge across it (see Materials and Methods) which would allow the circumoesophageal connectives to be pulled out laterally and pinned out of the way. Carrying out the GA technique on SOG that were then dried laterally onto the slides might help to ascertain the depth of the fluorescent cell in the funnel.

As in the previous section it was felt that perseverance with the intracellular injections would lead to morphological characterization of the dorsal unpaired neurone. But again it was also felt that these experiments ought to be temporarily shelved to allow electrophysiological and pharmacological aspects of the aminergic innervation to be investigated.

## CHAPTER 2

THE ELECTRICAL RESPONSE OF 2 IDENTIFIED INSECT MOTONEURONES  
TO PRESSURE EJECTED APPLICATION OF GLUTAMATE AND GABA OR  
CATECHOLAMINES.

SECTION I

THE EFFECT OF PRESSURE EJECTED GLUTAMATE AND GABA UPON THE  
ELECTRICAL RESPONSE OF AN IDENTIFIED METATHORACIC COXAL  
DEPRESSOR MOTONEURONE.

INTRODUCTION

In insects, most of the published electrophysiological experiments carried out to determine the effect of the putative central nervous system transmitter GABA, have used the DUM neurones and the giant interneurons of the cockroach sixth abdominal ganglion. It has been shown that that GABA is inhibitory in that it hyperpolarizes cells and depresses spontaneous activity: it causes a large increase in chloride conductance plus a smaller increase in the potassium one. This effect is inhibited by picrotoxin (Kerkut et al., 1969a and b; Pitman and Kerkut, 1970; Callec and Boistel, 1971b and c; Hue, Pelhate and Chanelet, 1979; Hue, Pelhate and Callec, 1981). Very little is known about the central effects of glutamate. (See General Introduction for a more extensive review of the evidence for the role of these amino acids in transmission.)

To learn more about the central effects of glutamate and GABA it was decided to use the identified coxal depressor motoneurone of the metathoracic ganglion of Periplaneta americana. This neurone is relatively large (about 80  $\mu$ m in diameter), is fairly easy to locate, and has been used by several workers to determine the central role of acetylcholine (see General Introduction). In addition, preliminary experiments had indicated that both these amino-acids cause a hyperpolarization of the cell body (Pitman, personal communication).

The experiments also served as an introduction to intracellular recording, with the expertise gained being of

great help when trying to ascertain the role of dopamine in the cockroach central nervous system by electrophysiological techniques.

MATERIALS AND METHODS

The experimental setup was essentially the same as that used for the intracellular dye injection experiments. The alterations are listed below.

The nerve cord from the prothoracic to the second abdominal ganglion was exposed and excised as described in Chapter I, Section I. It was mounted ventral side up on the black plastic slide with the metathoracic ganglion lying over the araldite ridge. This ganglion was desheathed and then the slide was transferred to the bath.

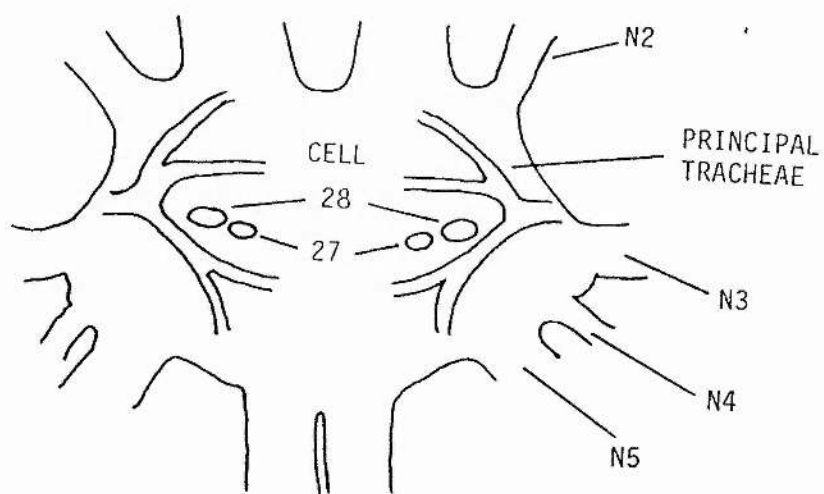
The soma of the metathoracic fast coxal depressor motoneurone, termed Df (Pearson and Iles, 1971), or cell 28 (Cohen and Jacklet, 1967) was located using two criteria. (1) The ventral side of the ganglion has a bilaterally symmetrical tracheal system in the shape of two V's. Cell 28 usually has a characteristic position with respect to this; lying near to the tip of the V (Fig. 2.1). (2) Another large cell, cell 27, lies medial and posterior to cell 28. These two cells are the largest in this area of the ganglion and are hence easy to locate. Using the above information cell 28 was usually fairly readily identified. Unwanted glial cells and connective tissue were removed as described previously (Chapter I, Section I).

The recording microelectrodes and holder were filled with 2M potassium acetate. Their resistance was usually 10-20 M ohms. Drugs were applied from microelectrodes by pressure ejection. These were filled with either 0.1 M or 0.01 M gamma-aminobutyric acid (GABA; BDH) or 0.1 M sodium



Figure 2.1 A diagram of a metathoracic ganglion showing the position of neurones 27 and 28 in relation to the principle tracheae and the peripheral nerves.

ANTERIOR  
CONNECTIVES



POSTERIOR  
CONNECTIVES

glutamate (BDH). The solutions were made up in saline.

In these experiments the amplifier/bridge balance circuit was constantly in the recording mode. For the experiments using only one recording microelectrode, current was passed through the microelectrode by way of the amplifier circuitry using a Grass S44 stimulator as the current source. When two recording microelectrodes were used, one was connected as before to the amplifier, with current being passed through it from the D.C. source in the amplifier. The other was connected to the Grass stimulator through a 100 Mohm resistor and another D.C. current source. This enabled the cell to be polarized as well as pulsed through the second electrode.

Impalement with one microelectrode was accomplished as described in Chapter I, Section II. When two microelectrodes were being used, the first penetration was carried out as above except that the microelectrode was held at about  $30^{\circ}$  to the vertical and was brought to one side of the cell. The second microelectrode was then connected to the stimulator and brought in at  $30^{\circ}$  to the other side of the cell. For both microelectrodes 25-30 nA, 20 msec duration, negative current pulse were used to bring about the penetration. As the second electrode approached the cell surface small deflections were seen on the oscilloscope trace of the first electrode. As it started to push into the cell surface the deflections were seen clearly as small hyperpolarizing pulses. When penetration occurred the pulses suddenly increased to the

size determined by the neuronal input resistance. The pulsing was immediately switched off and the cell allowed to recover. When one microelectrode was used the experiment was continued if the cell's membrane potential was at least  $-65$  mV. With two microelectrodes a cell with a membrane potential of at least  $-60$  mV was considered worth using.

The GABA or sodium glutamate was ejected from the microelectrode using a General Valve Corporation Picospritzer II, with air pressure provided by a Killaspray garden spray pressure tank (ASL airflow Ltd.). Using a Prior micromanipulator the drug microelectrode was positioned near to the cell surface between the other two microelectrodes. The pressure of the ejection pulses was kept constant at 15 psi and the quantity of drug applied adjusted by varying the pulse duration. Unlike iontophoresis, there is no way of preventing diffusion of drugs from the tip of pressure ejection electrodes. This could cause desensitization of a response which must be avoided, especially when the effectiveness of various inhibitors is being tested. Hence it was always checked that the magnitude of a response remained constant during a control period of several minutes before any other drugs were used.

Some of the drugs used - picrotoxin (Sigma), flurazepam (Roche products), 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid (SITS; BDH) - were added directly to the bath. The position of the suction tube in the bath (see Fig.1.30) was adjusted so that the saline volume in the

bath was 6 mls. The drugs were slowly and carefully added using a Gilson pippette. Drugs were made up as concentrated stock solutions so that no more than 10-200  $\mu$ ls was added at the one time to the bath. Picrotoxin, flurazepam, and SITS were made up in saline to concentrations of 1.7 mM, 10 mM and 1mM respectively. Fresh solutions of drugs were made up daily.

RESULTS

Pitman and Kerkut (1970) estimated that the reversal potential of the GABA response in cockroach sixth abdominal ganglion DUM neurones was about -79 mV.

In this series of experiments a similar value was obtained for the GABA response of Df (Fig. 2.2). This reversal potential value was also obtained with glutamate (Fig. 2.3).

Although the response to each drug does vary between preparations, it was found that for any one cell the sensitivity to GABA was greater than to glutamate. In most experiments, although the concentration of glutamate in the pressure ejecting electrode was ten fold greater, a longer pulse of glutamate was still required to produce an equivalent response to that of GABA. Also, in several preparations in which Df gave a large GABA response very little response to glutamate was obtained even when the cell was hyperpolarized.

The response to both GABA (Fig. 2.4) and glutamate (Fig. 2.5) was rapidly and reversibly blocked by low concentrations of picrotoxin. The GABA response was more sensitive to this drug: 8  $\mu$ M in the bath was sufficient to greatly reduce the response after 3-5 min; for glutamate 80  $\mu$ M was required.

One of the most interesting results came from prolonged exposure of the cell to picrotoxin. In insects, most motoneurone cell bodies do not support action potentials; depolarization produces a series of damped oscillations

which increase in size as the depolarization is increased (Pitman, 1979 and 1984; Fig.2.6). Pitman (1979) suggested that these might result from the combination of a high level of inactivation of the inward current and a fairly large and early increase in the delayed outward current. In some Df preparations it is also possible to record small spontaneous excitatory potentials (2-5 mV); these are thought to result from the passive spread of current from more active regions of the neurone (Pitman et al., 1971). For the picrotoxin experiments it was usual to start washing off the drug between 1-5 min after its application, otherwise it was difficult to bring the response back to the original level. On prolonging the exposure it was first noticed that the spontaneous excitatory postsynaptic potentials increased in size and frequency. At about 16-20 min some of the excitatory potentials were up to about 20 mV. If left still longer, even larger (up to 40mV), long lasting depolarizations occurred (Fig. 2.7); their size and frequency increasing as the cell was depolarized (Fig. 2.8). The final result for some specimens was that the cell started to fire action potentials, either spontaneously or in response to depolarizing pulses (Fig. 2.9a). Action potential frequency was increased by increasing the pulse size (Fig. 2.9b) and the cell adapted if the pulsing was maintained (Fig. 2.9c) or if its membrane potential was kept at a depolarized level (Fig.2.14a). It is worth noting that in two of the experiments the picrotoxin had been at least partially washed off before it was discovered the cell could support action potentials.



A variety of cells, both neuronal and non neuronal, contain a sodium driven, chloride-bicarbonate exchange mechanism which is involved in intracellular pH regulation; it is inhibited by SITS and related compounds (Kuffler et al., 1984). Three types of response were produced by prolonged exposure to this drug:

(i) SITS at both 20 and 100  $\mu$ M, produced either a negligible or a small reversible decrease in the cell's response to GABA. There was no change in the cell's response to depolarizing pulses even after 1.25 hr.

(ii) 100  $\mu$ M SITS produced slight reduction in the GABA response. After 25 min, however, the cell no longer behaved in a passive fashion to depolarizing pulses; an active component, that could last longer than the pulse, came into play (Fig. 2.10). No action potentials could be produced even after an hour.

(iii) 100  $\mu$ M SITS again only slightly affected the GABA response, but this time the cell produced action potentials in response to depolarizing pulses (Fig. 2.11). The preparation had been well washed before the action potentials were seen.

During these experiments the cells were constantly stimulated with 250 msec duration, 10 mV hyperpolarizing pulses at a rate of 1 or 2 per sec. The membrane resistance of the cell could therefore be monitored; a decrease in the pulse size indicating a decrease in membrane resistance. Both GABA and glutamate produced this result (see Fig. 2.2 and 2.3). It also allowed the response to be measured at two different membrane potentials. At potentials near the

reversal potential for the glutamate and GABA responses, there was a hyperpolarization at the less negative value of the pulse and a depolarization at the other. In theory these potential changes should follow the same time course, but for many cells it was found that the hyperpolarizing response to GABA peaked before the depolarizing one (Fig. 2.12). The opposite result (that is the depolarization peaking before the hyperpolarization) was obtained with some cells in response to glutamate (Fig. 2.13). This may suggest that there are distinct receptors for GABA and glutamate: each having a higher affinity for one of the drugs but in addition being able to interact with the other drug as well (see Discussion).

Over the last few years evidence has been collected for the presence of specific benzodiazapine receptors in the mammalian brain (see short review by Mohler, 1982). The main central effects of benzodiazapines, such as their hypnotic, anticonvulsant and muscle relaxant effects, appear to be connected with the enhancement of transmission at certain GABA synapses (Mohler, 1982). No consistent results were obtained with the benzodiazapine Fluorezepam at concentrations up to 1 mM in the bath. This suggests, therefore, that *Df* does not have the appropriate receptors, although they still could be present on other neurones.

Figure 2.2 (a)-(c) Effect of depolarizing the neuronal membrane upon the response to pressure ejected GABA ( $\triangleright$ ; 10 mM, 200 ms 15 psi). The cell was stimulated with 10 mV; 250 ms duration, hyperpolarizing pulses at a rate of 1 per sec.

(a) 20 mV depolarized.

(b) Resting potential (-76 mV).

(c) 20 mV hyperpolarized.

It may be seen that the reversal potential for the GABA response is slightly more negative than the resting potential of the cell. Note that at -76 mV the response was a small hyperpolarization followed by small depolarization ( $\triangleright$ , see end of results).

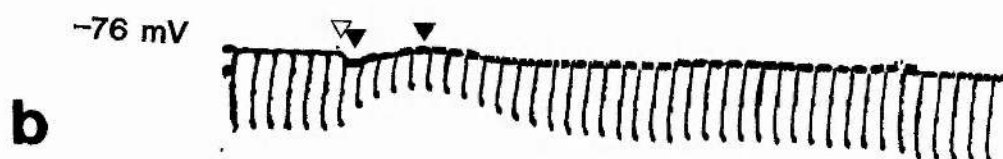
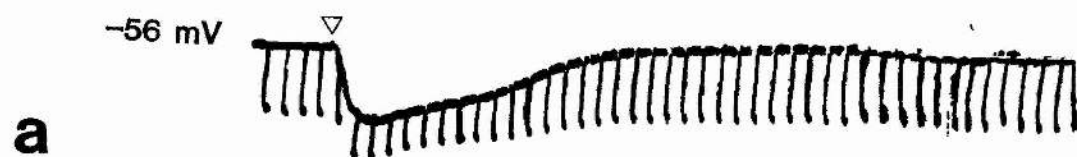
Figure 2.3 (a)-(c) Effect of hyperpolarizing and depolarizing the neuronal membrane upon the response to pressure ejected glutamate ( $\triangleright$ ; 100 mM; 150 ms 15 psi). The cell was stimulated as in Fig. 2.2 at a rate of 2 per sec.

(a) 6 mV depolarized.

(b) Resting potential (-74 mV).

(c) 6 mV hyperpolarized.

By comparing (a) and (b) it may be seen that the response reverses between -74 and -78 mV. Note that in both (a) and (b) it may be seen that the depolarizing part of the response reaches its peak before the hyperpolarizing part ( $\triangleright$ , see end of results).



10 mV  
5 sec

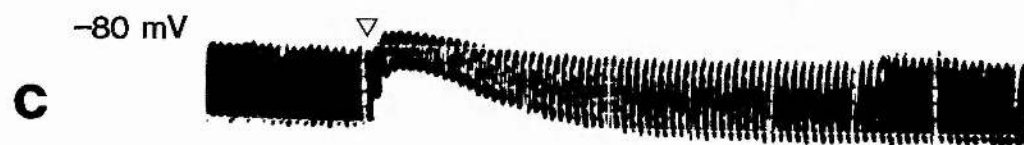
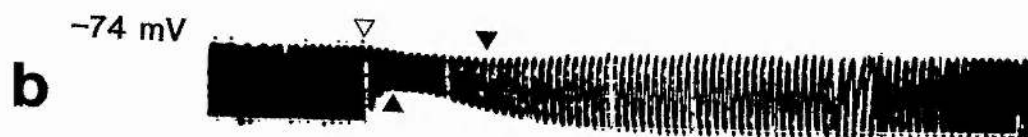


Figure 2.4 (a)-(e) Effect of picrotoxin upon the response to pressure ejected GABA (►; 10 mM. 50 ms, 15 psi). The neurone was stimulated as in Fig. 2.2.

(a) Normal response.

(b) 1 min after adding picrotoxin (final concentration 8  $\mu$ M) to the bathing solution.

(c) Response after a 3 min wash.

(d) and (e) Response after washing for 9 and 14 min respectively.

It may be seen that the membrane resistance of the cell increased after the addition of picrotoxin (b). Since after washing, however, it did not decrease to the original value the <sup>apparent</sup> increase may have been due to increased electrode resistance.



10 mV  
5 sec

Figure 2.5 (a)-(c) Effect of picrotoxin upon the response of pressure ejected glutamate (►, 100 mM, 50 ms. 15 psi). The neurone was stimulated as in Fig. 2.2.

(a) Normal response.

(b) 3 min after adding picrotoxin (final concentration 80  $\mu$ M) to the bathing solution.

(c) Response after a 10 min wash.

(▷) indicates a small excitatory potential. These became larger and more frequent after prolonged application of picrotoxin (see later results).



10 mV |  
5 sec



Figure 2.6 Damped membrane oscillations (bottom trace) produced by a 20 nA depolarizing pulse (top trace) through the second electrode. The cell had been hyperpolarized by 30 mV from its resting potential of 70 mV.

Figure 2.7 Spontaneous activity of a cell 35 min after the addition of picrotoxin (final concentration 8  $\mu$ M) to the bath. The cell had been washed for 5 min before the record was made. The cell was stimulated as in Fig. 2.3.

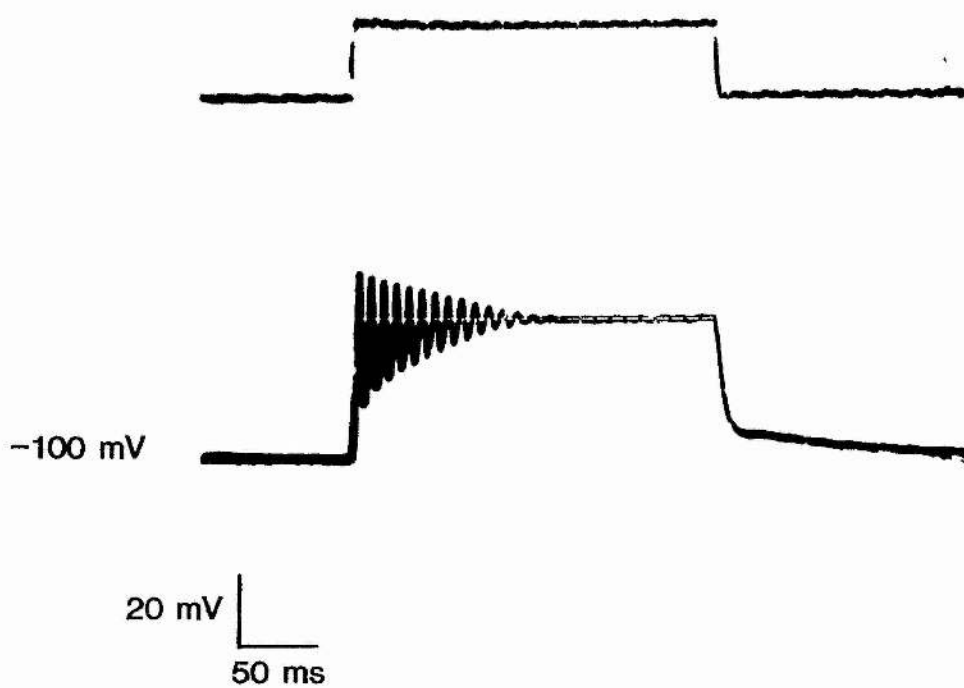
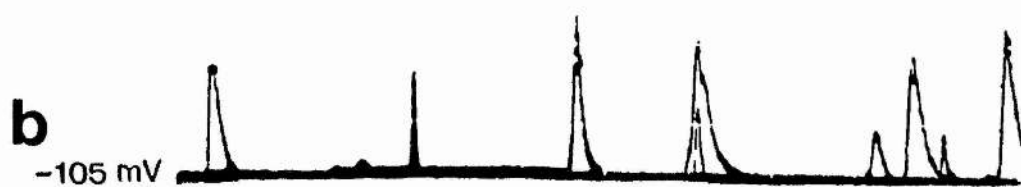
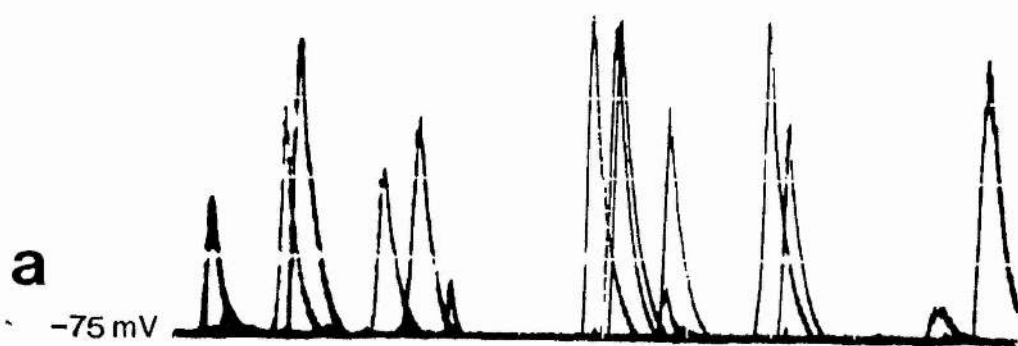


Figure 2.8 (a) and (b) Spontaneous activity of a cell after prolonged (30 min) exposure to picrotoxin (final concentration 8  $\mu$ M) at -75 mV (a) and -105 mV (b). Note that (i) the pulses are of varying size, (ii) are up to 500 ms in duration, and (iii) are smaller and less frequent when the cell is hyperpolarized.



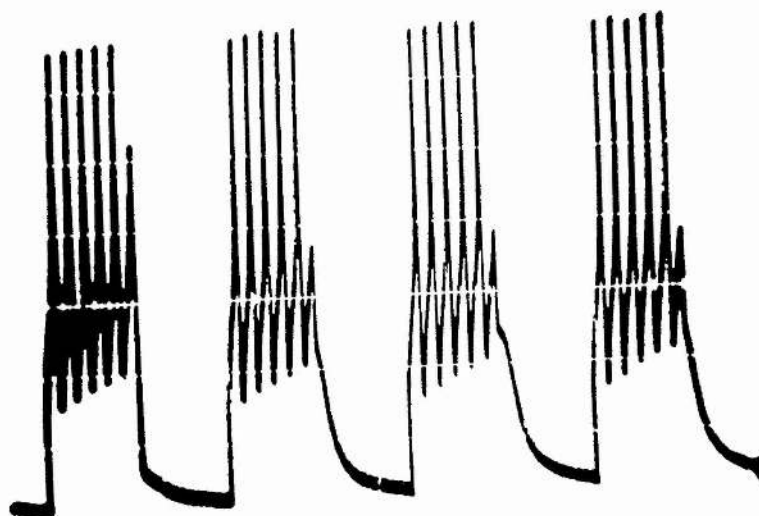
10 mV

500ms

a



b



10 mV  
200 ms

c

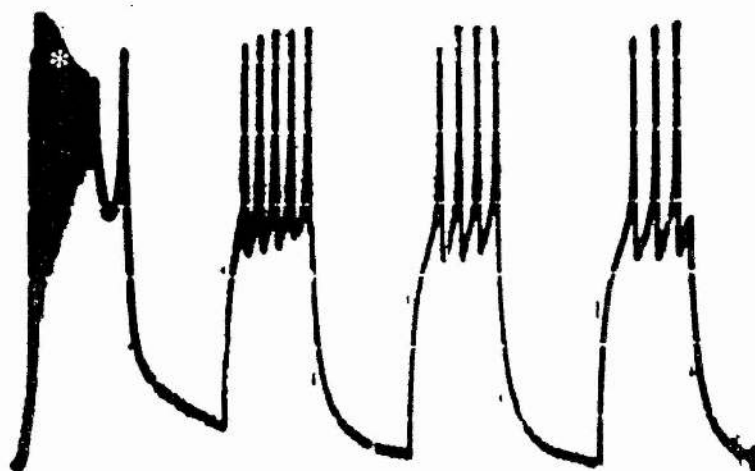


Figure 2.9 (a)-(c)

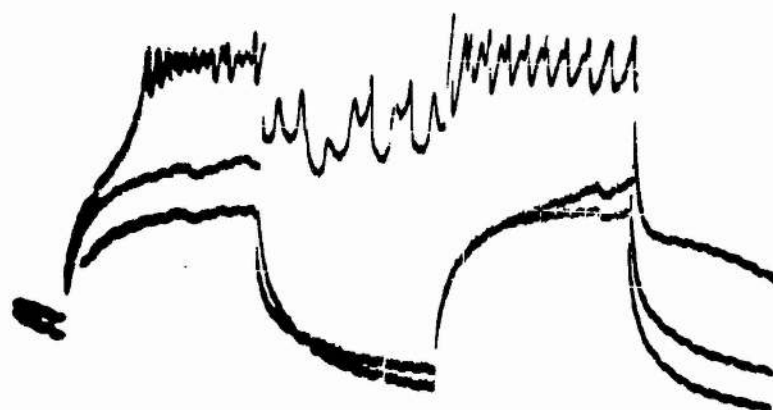
(a) Spontaneous regenerating activity of the neurone after prolonged exposure (40 min) to picrotoxin (final concentration 8  $\mu$ M). The neurone was stimulated with hyperpolarizing pulses as in as in Fig. 2.3.

(b) Increased activity of the neurone in response to a -10 mV, 250 ms depolarizing pulse.

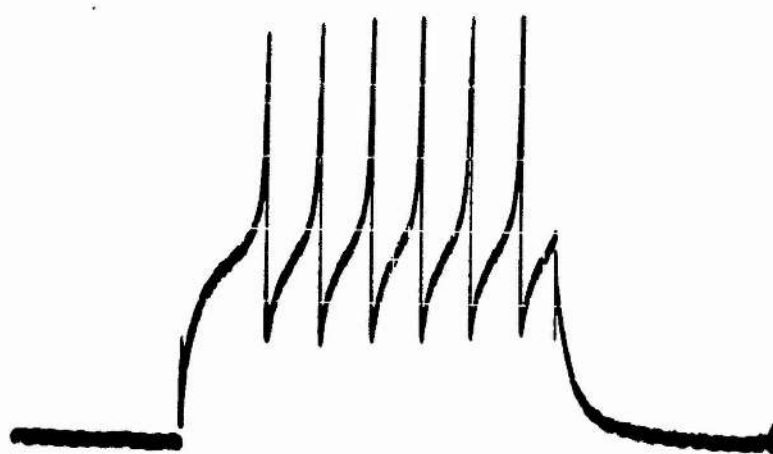
(c) Decreased activity due to adaption of the neurone as a result of repeating depolarizing pulses.

Figure 2.10 Activity of a cell in response to depolarizing pulses after a 30 min exposure to 100  $\mu$ M SITS. Note that the response was not always passive and that it could last longer than the pulse duration.

Figure 2.11 Regenerating activity of a the neurone in response to depolarizing pulses. The cell had been exposed to 100  $\mu$ M SITS for 30 min and then washed for 20 min. This record was made 10 min after the washing had stopped.



10 mV  
100 ms



10 mV  
50 ms



Figure 2.12 Response of a cell to pressure ejected GABA (▷, 10 mM, 50 ms, 15 psi). The neurone was stimulated as in Fig. 2.2. Note that the hyperpolarizing and depolarizing (▶) responses have different time courses.

Figure 2.13 Response of the neurone to pressure ejected glutamate (▷, 100 mM, 100 ms, 15 psi). The cell was pulsed as in Fig. 2.2. It may be seen that as in the above figure the hyperpolarizing and depolarizing parts of the response peak at different times (▶), but here the order is reversed (see also Fig. 2.3).



10 mV  
5 sec



10 mV  
5 sec

Figure 2.14 (a) and (b)

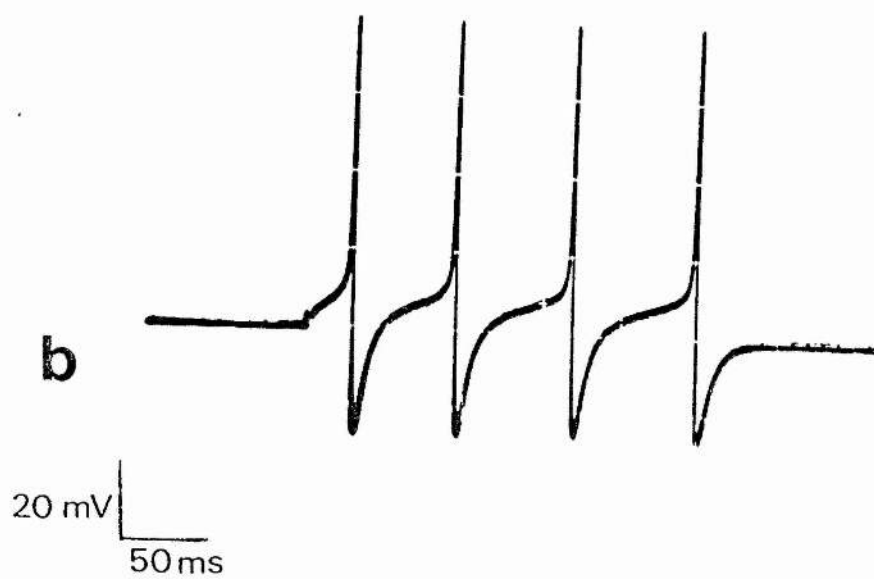
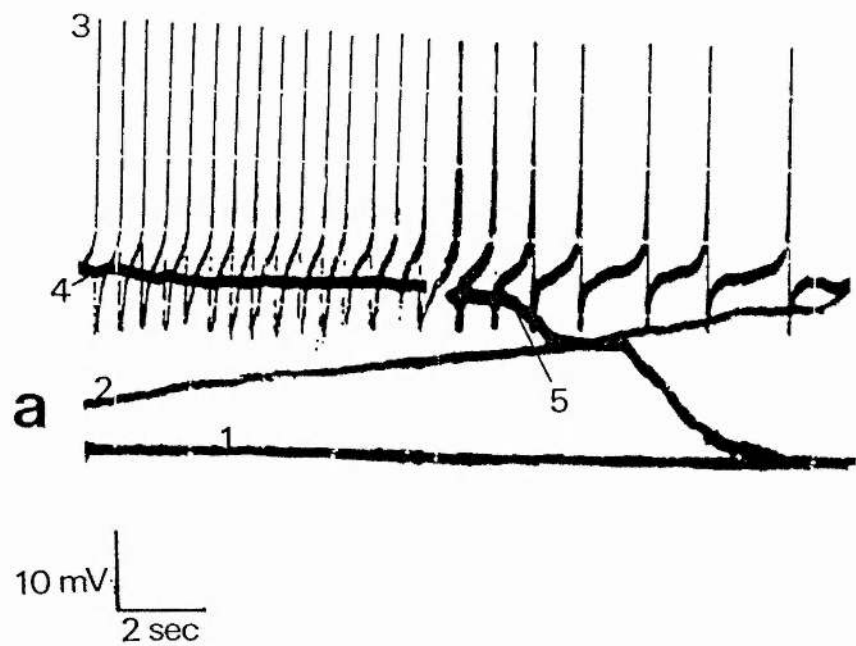
(a) Spontaneous action potential production recorded in the cell body of Df after prolonged exposure (40 min) to picrotoxin (final concentration  $8\text{ }\mu\text{M}$ ). 4 sweeps of the oscilloscope are shown.

(1) 72 mV resting potential of the neurone.

(2) The neurone was slowly depolarized.

(3) At about 20 mV depolarization action potentials were recorded. Not that their frequency decreased and finally ceased (as seen in (4)) showing that the cell had adapted to the depolarization. The cell was returned to its resting potential (5).

(b) Large overshooting action potentials recorded from a prothoracic ganglion DUM neurone in response to a 250 ms depolarizing pulse.



DISCUSSION

Due to the similarity in the reversal potential of the GABA response and the effect of picrotoxin on both Df and DUM neurones (see Pitman and Kerkut, 1970), it is highly likely that the ionic mechanisms involved are identical in these cells. Nothing can be said, however, about the receptors of the cells since picrotoxin may block chloride channels, not receptors, and the work on acetylcholine, for example, has provided evidence for three different receptors in the insect central nervous system, each responding in an indential fashion to applied acetylcholine (see General Introduction).

The same results were obtained for glutamate on Df, except that the cells were less sensitive. It is therefore possible that glutamate was acting at GABA receptors. If this were the case it would certainly be very different from the situation found at the insect neuromuscular junction, where GABA is thought to be an inhibitory transmitter and glutamate an excitatory one (Usherwood, 1978). Hence the results suggest that the peripheral and central actions of the two drugs must be different. Since glutamate is released by excitatory motoneurones at the neuromuscular junction, one would expect it to be released from the central branches of these neurones as well and to act at its own specific receptors. It could be, therefore, that Df does not have glutamate receptors and that the configuration of the GABA receptors is such that glutamate can act as an agonist; or that there are glutamate receptors which act by the same ionic mechanism as the GABA receptors on Df, but are fewer

in number and/or are concentrated on less accessible parts of the cell body surface. Thomas (1984) determined that the membrane capacitance of the cell body is 10  $\mu\text{F}$  per sq. cm with respect to the apparent surface area of the cell body. Since this value is relatively high (the membrane capacitance of a squid axon is 1.1  $\mu\text{F}$  per sq. cm) he suggested that the cell membrane is considerably infolded.

Pressure ejecting one drug in the presence of a saturating concentration of the other would help to clarify the situation. The use of specific agonists and antagonists would be also very useful. The isoquinoline alkaloid, bicuculline, has been found to be a relatively specific GABA inhibitor in mammalian central nervous system (Curtis, Duggan, Felix and Johnston, 1970). N-methyl-D-aspartate, quisqualate and kainate are potent glutamate agonists in rat hippocampus (Crunelli, Forda, Collingridge and Kelly, 1982; Collingridge, Kehl and McLennan, 1983). 2-amino-4-phosphobutyric acid has been shown to be a potent glutamate antagonist on locust muscle (Cull-Candy, Donnellan, James and Lant, 1976). In the mammalian central nervous system specific glutamate inhibitors include:  $\gamma$ -D-glutamylglycine; DL-2-amino-5 phosphovalerate; and D- $\alpha$ -aminoadipate (Crunelli et al, 1982; Collingridge et al, 1983; Crunelli, Forda and Kelly, 1984). All of the above drugs may be of use in helping to distinguish between the glutamate and GABA responses on Df although it must be remembered that for most their specificity in the insect is unknown.

As mentioned previously most insect motoneurons do not

generate action potentials. In a few of the large ventral motoneurons of the cockroach, for example the common inhibitory neurone (Pearson and Fourtner, 1973), small spikes are seen (2-10 mV). These, like the small excitatory potentials sometimes seen in Df, are thought to be due to passive electrotonic spread from electrically active regions of the axon.

In the experiments described here prolonged exposure to both picrotoxin and SITS caused the production of actions potentials or large non-regenerative depolarizations from Df in some specimens.

Picrotoxin is thought to be a chloride channel blocker. As Df's exposure to this drug lengthened it became progressively more excitable; the spontaneous non-regenerative excitatory potentials increased in size, frequency and duration. The blocking of the GABA and glutamate responses took place within a few minutes, before any substantial changes in cell excitability had taken place. This implies that either picrotoxin acts at another site in the membrane (for which it has a low affinity), or that the long-term changes in intracellular chloride ion concentration may directly or indirectly (perhaps by affecting other intracellular ion concentrations) increase cell excitability. Since for one of the specimens the picrotoxin had been washed off before the cell started firing action potentials, it suggests that the picrotoxin itself is not directly responsible for the effect, unless of course the picrotoxin acts by being slowly but irreversibly bound to some part of the membrane or internalized.

SITS is an inhibitor of sodium driven chloride-bicarbonate exchange (the chloride is driven out and the bicarbonate in, both against their concentration gradients). As after exposure to picrotoxin action potentials could also be observed after SITS had been washed off and so again it does not appear to be directly responsible for the result (with the same objection as above). Unlike picrotoxin, no increase in spontaneous synaptic activity of Df was seen in any of the preparations. In the case of the picrotoxin experiments the increase may have been due to this drug causing a general increase in the excitability of the preparation (through antagonism of any chloride dependent inhibitory responses), although it must be said that in each case no spontaneous postsynaptic excitatory potentials were seen at the start of the SITS experiments before drug addition.

There are also certain other conditions under which Df can support action potentials. Within four days after either cutting the axon or giving an injection of colchicine, overshooting action potentials can be recorded from Df; the increased electrical activity being correlated with cytological changes (Pitman, Tweedle and Cohen, 1972). Prolonged exposure of cockroaches to carbon dioxide or nitrogen and prolonged exposure of the nervous system to anticholinesterase can also result in action potential recordings from some specimens (Pitman, personal communication). In the case of the colchicine pretreatment, Pitman (1975) found that 10 nM tetrodotoxin (a sodium channel blocker) reversibly depressed the action potentials. Hence he concluded that sodium ions carry most of the inward



current of the action potential.

Pitman (1977) was also able to induce action potentials in Df by intracellularly injecting citrate ions or EGTA (both calcium chelators) or by externally applying tetraethylammonium ions (TEA; suppresses the delayed potassium current of action potentials). Both TEA and intracellular citrate also increased the input resistance and time constant of the motoneurone. This time it was calcium ions which carried the inward current. Pitman (1977) concluded that the initial decrease in internal free calcium concentration caused by citrate would reduce both the resting membrane conductance and the delayed potassium current and result in action potential production; but that TEA would work by blocking potassium channels directly, thereby unmasking the voltage dependent calcium channels.

In support of this Thomas (1984) injected calcium ions into voltage clamped Dfs. He found that, as well as the (voltage dependent) delayed potassium current, there is also a calcium-mediated potassium current; the intracellular accumulation of calcium rather than the inward calcium current itself being the important factor. The rapid activation of this calcium-mediated potassium conductance prevents the inward calcium current from causing a regenerative depolarization. He concluded that a blockage of the outward current could cause the cell to become electrically excitable.

From the above it is seen that picrotoxin and SITS could produce action potentials by one of (at least) two

mechanisms. To distinguish between them the effect of the following could be tried:

- (i) Using sodium or calcium free saline once action potential production had been achieved, to see if under either of these conditions it was abolished.
- (ii) The addition of tetrodotoxin to the bath.
- (iii) Using saline containing inorganic or organic calcium channel blockers.

If sodium carries the inward current then picrotoxin and/or SITS must be able to activate these channels; if the current carrier is calcium, then this current must be greatly increased either directly or through blockage of the calcium-mediated potassium current.

It is worth noting, however, that unlike citrate, TEA and colchicine the action potentials produced by both SITS and picrotoxin were not overshooting; their maximum size was 50 mV. Two possibilities about action potential origin therefore arise: they could be generated by (i) active propagation in the soma or (ii) by passive spread from an active region in the cell (this may account for their non-overshooting nature). On comparison of the results obtained here with those of other workers, however, the second suggestion seems unlikely (see below). Fig. 2.14a shows the result of depolarizing a picrotoxin treated preparation. After 20 mV depolarization, action potentials are seen; their frequency is high to begin with but then decreases as the cell adapts and then finally ceases. Although the action potentials are only 30 mV in height they are very similar in shape to those recorded from the cell body of cockroach neurones in which

full sized action potentials are consistently observed (Fig. 2.14b). Antidromic stimulation of Df in specimens that had been pretreated with colchicine resulted in the recording of action potentials in the cell body (Pitman et al, 1972). By increasing the stimulus frequency these authors found that the action potentials could be separated into two components: (i) a slow, small (up to 15 mV), graded response and (ii) a larger, rapidly rising and overshooting late component which disappears at high stimulation rates. They suggested that the slowly rising phase represented electrotonic spread of an action potential from the spike initiation zone into the cell body; and the rapidly rising phase an active invasion of the soma. In addition, Edwards and Mulloney (1984) produced a model of the electrotonic structure of an identified motoneurone in the stomatogastric ganglion of the spiny lobster. With this they are able to predict the passive response of the cell to injected current and have shown that a 92 mV, 1 ms, action potential in the axon is attenuated to a 6 mV, 8 ms impulse in the soma, the rise time of which is greatly increased compared to the original. From the above, therefore one might expect that the action potentials recorded in the picrotoxin and SITS experiments would be small (less than 20 mV) and slowly rising if they resulted from the passive spread of current. This<sup>is</sup> clearly not the case and so it may be concluded that<sup>it</sup> is more likely that they arise through active invasion of the soma. In conjunction with this is the suggestion that for this active invasion to occur both picrotoxin and SITS must alter the cell body properties of Df.

Questions do arise, however, with the above conclusion about action potential production in both the picrotoxin and SITS experiments and these will now be discussed in the following paragraphs.

In all the picrotoxin treated specimens which could support action potentials large (up to 40 mV), spontaneous and relatively long lasting (up to 500 ms) depolarizations were seen as a prerequisite to action potential production. One possible mechanism is that picrotoxin causes trains of impulses to be produced at the spike initiation zone (or else where in the dendritic tree) by, as stated before, a general decrease in inhibition throughout the preparation and that the electrotonic spread from these summate to produce a large long lasting depolarization in the cell body. Since this is so different from the normal response it seems sensible to suggest that, as with the active production of action potentials, the cell body properties must also have changed. Hyperpolarization of the soma may be transmitted to the site(s) of spike initiation, this would reduce action potential production and hence the maximum size and frequency of the excitatory potentials seen in the soma (as seen in Fig. 2.8).

Another explanation, however, which fits in with the idea that the spikes actively invade the soma, is as follows. On comparing Figs. 2.9c and 2.7 it may be seen that the maximum height of the first group of action potentials (Fig. 2.9c\*) decays in a similar fashion to that of the large, long lasting depolarization (Fig. 2.7). This suggests that the

mechanisms behind this decay may be similar. From this it can be postulated that the changes in cell properties which result in action potential production occur in two stages: (i) The inward current is no longer rapidly inactivated and the early rising outward current is greatly diminished. This would result in impulses whose shapes are determined by the activation/inactivation pattern of the inward current channels with respect to time.

(ii) An outward current appears which activates with a similar time course to that found in neurones (for example the DUM neurones) which normally do support action potentials. This would decrease the time course of a single impulse by increasing the rate of repolarization and also allow trains of impulses to be produced by limiting the degree of inactivation of the inward current channels.

Hyperpolarization of the cell body reduced both the maximum size and the frequency of the spontaneous potentials (Fig. 2.8). If all the potentials recorded were produced actively in the cell body one would expect that on hyperpolarization their frequency would decrease but not their size (which if anything should increase due to an increased drive on the inward current). To account for this one must postulate that the potentials originate from at least two sources:

(i) Active invasion of the the cell body - this would result in the large potentials. These would disappear on hyperpolarization.

(ii) Electrotonic spread from the axon - this would result in the smaller potentials (less than 20 mV). At normal potentials certain of these would large<sup>be</sup> enough to initiate

an active invasion of the soma and result in the production of a large long lasting potential or an action potential. This would not occur when the cell body was hyperpolarized above a certain level (as was seen in Fig. 2.8).

In certain SITS preparations stimulation produced long lasting depolarizations which had small (2-10 mV) excitatory potentials superimposed upon them (Fig. 2.10). No large spontaneous depolarizations were seen. Perhaps the most plausible explanation is to consider that this response results from the sum of two distinct responses:

(i) A long lasting depolarizing potential is produced in the cell body by stimulation bringing about the switching on of a slowly inactivating inward current. It may be similar to the one seen in the picrotoxin experiments. In addition, as was also suggested for picrotoxin, any early rise in the outward current would also have to be greatly decreased. Evidence from two groups of workers suggests that this current may be due to sodium. Eaton and Brodwick (1979) and Nonner, Spalding and Hille (1980) reported that internal application of SITS removes sodium inactivation in squid axon and at frog muscle respectively. Nonner et al. (1980) also stated that both external and internal SITS depressed the sodium current of the action potential which may explain why the action potentials seen in Fig. 2.11 were not overshooting.

(ii) Small excitatory potentials, which originate from action potentials produced elsewhere in the cell as a result of the spread of the depolarizing current from the cell body. These spread passively to the cell body and give the



fluctuations in the long lasting potential.

As discussed above for picrotoxin, the switching on of a delayed outward current in addition to the inward current may lead to action potential production in the cell body.

Experiments to ascertain the ions involved in the action potential production were suggested above. Since externally applied TEA alone can produced calcium dependent action potentials (Pitman, 1975) it would not be possible to use this to determine the role of a delayed potassium current in the production of the long lasting potentials and the action potentials. A worthwhile set of experiments would be to monitor the activity in nerve 5r1 (the peripheral projection of Df), in specimens which showed either the large potentials and/or action potentials, at various membrane potentials with or without antidromic and orthodromic stimulation of the cell body. By correlating the activity in the nerve and the cell body it may be possible to determine the origin of the impulses in the cell body (that is, do they arise through invasion of, or de novo production in, the soma) and to ascertain whether those that invade the soma do so actively or through the passive spread of current.

In conclusion, it appears that for both picrotoxin and SITS treated preparations there is an alteration in the membrane properties of the cells which enables them to actively support action potentials, but only in the case of picrotoxin is there a production of large spontaneous excitatory potentials. It has been posulated that it is more

likely that the potentials arise through an active mechanism in the soma, although the results of the experiments suggested above would be required before any real conclusions about the mechanism and the ions involved could be drawn.

It was stated in the results that when  $D_f$  was pulsed near its reversal potential for the GABA response, the small hyperpolarizing and depolarizing components of the response peaked at different times in some preparations (Fig. 2.12). This discrepancy between peak times is also seen when comparing equivalent hyperpolarizing and depolarizing responses at higher levels of polarization (see Fig. 2.2, top and bottom traces). In fact, examination of the trace at -76 mV in Fig. 2.2 shows that at this one potential there was first a small hyperpolarization and then a small depolarization.

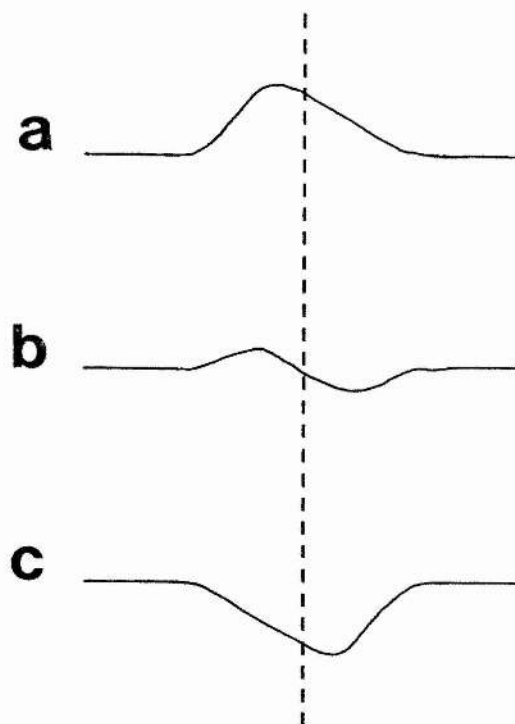
Pitman (personal communication) found that when the cell was voltage clamped at the GABA response reversal potential there was a small hyperpolarizing current followed by a small depolarizing current. As above, on comparison of responses at equivalent potentials slightly less and more negative than the reversal potential, he found that the current always peaks sooner when it is hyperpolarizing (Fig. 2.15); the two currents becoming nearer the mirror image of one another as the degree of polarization increased.

There are a few possible explanations:

(i) There could be two receptors, one being associated with potassium channels, the other with chloride channels. This



Figure 2.15 Diagram of the transmembrane currents obtained on pressure ejecting GABA at the membrane potentials (a) slightly more negative than (b) equal to and (c) slightly more positive than the reversal potential for the GABA response. Note that at the reversal potential (b) there is a small outward, followed by a small inward current. Note also the different peak times for the three responses.



OUTWARD CURRENT  
↑  
↓  
INWARD CURRENT

would explain the slightly different reversal potentials. If the receptors had different affinities for GABA, this would account for the different response times. However, both responses were seen to be blocked by picrotoxin at fairly low concentrations (10 ug/ml) which would be unlikely to affect any potassium channels, unless by some, as yet unknown, indirect action.

(ii) If there is only one type of receptor and one ion involved but in addition there exists an intracellular ion concentration gradient along the length of the cell, then this would result in different reversal potentials at different points. To achieve the different response times for the depol- and hyperpolarization, the axon of the cell would have to be less accessible to the GABA. If the receptors were equally accessible then one would expect the responses to cancel one another out so that the total response near the reversal potential would be negligible. Once the membrane potential gradient along the cell body and the initial part of the axon was such that both responses were either depol- or hyperpolarizing then they would be additive. If the receptors around the initial part of the axon remained inaccessible then only the first part of the response would be seen. These latter two points could explain why the biphasic response was not seen in every preparation. Hence recording from just beneath the cell surface when the cell was depolarized from the GABA reversal potential, may result in a small hyperpolarization (produced by the nearby receptors), followed by a small depolarization (produced by receptors at the axon end of the cell and along

the initial axon segment). In support of this, it has been reported that the response to GABA in rat hippocampus CA1 pyramidal cells is hyperpolarizing at the cell body, but depolarizing at the dendrites (Anderson, Dingledine, Gjerstad, Langmoen and Laursen, 1980; Blaxter and Cottrell, 1985). Anderson et al.<sup>(1980)</sup> suggested that the depolarizing action of GABA was probably not mediated through chloride but through an increased conductance to other ions, notably sodium). Blaxter and Cottrell<sup>(1985)</sup>, however, showed that the depolarization in the dendrites was chloride dependent which supported the other suggestion by Anderson et al. (which they considered less likely) that the intracellular chloride concentration is higher in the dendrites compared with the soma

(iii) It is also possible that there is one type of receptor which can be associated with either one of two distinct chloride channels and that these have slightly different reversal potentials, due to their slightly differing permeabilities to other ions. As before, one group of chloride channels would have to be further away from the recording electrode and/or the receptors involved less accessible to the GABA. In support of this Benoit, Corbier and Dubois (1985) have found that at frog nodes of Ranvier there are two different sodium channels associated with the action potential. Their activation/inactivation kinetics and voltage dependences are slightly different and this results in different reversal potentials and time courses for the two currents.

(iv) Finally, it was noted in the results that some specimens also gave a biphasic response near the reversal

potential of the glutamate response, but that order of the polarizations were reversed (that is, the hyperpolarization occurred before the depolarization). It is therefore possible that glutamate and GABA can act at each others receptors, with the response time for acting at their own receptors being quicker. In this case the reversal potentials for the GABA and glutamate responses would need to be very similar but not identical. At membrane potentials away from the reversal potentials only one response phase would be seen provided that the time each drug took to act the other's receptors was short. Since most of the transmitter released at a genuine synapse is contained within the synaptic cleft there is little chance of a transmitter being able to reach a second synapse. Hence, no problems would be caused by the ability of glutamate and GABA to activate each others receptors. In the mammalian peripheral nervous system, for example, it is known that externally applied dopamine can act at adrenoceptors as well as at its own specific ones (Golberg and Kohli, 1979).

## SECTION II

THE EFFECT OF PRESSURE EJECTED APPLICATION OF BIOGENIC  
AMINES UPON THE ELECTRICAL RESPONSE OF AN IDENTIFIED  
PROTHORACIC INHIBITORY MOTONEURONE.

INTRODUCTION

It has been believed for several years now that the biogenic amines (dopamine, noradrenaline, adrenaline and serotonin) act as neurotransmitters in the vertebrate central nervous system (Krnjevic, 1974; Fuller, 1982). More recently a neuroendocrine role for dopamine in the anterior pituitary (Gudelsky, 1981) and a central neuromodulatory role for both noradrenaline and serotonin (Dismukes, 1977b) have been proposed. Noradrenaline is also released from the terminals of peripheral sympathetic neurones where it serves as a neurotransmitter or local neurohormone, acting on smooth and cardiac muscle and certain glands (Smith, 1973). It is now known that dopamine too has peripheral actions. It causes smooth muscle relaxation and inhibition of noradrenaline release from postganglionic sympathetic nerves (Golberg and Kohli, 1983). Adrenaline, which is released from the adrenal medulla, acts as a true hormone and has a number of effects throughout the body (Douglas and Rubin, 1963). At the moment there is little evidence on the role of octopamine in the vertebrate nervous system.

Both adrenaline and noradrenaline exert their effect through so called adrenoceptors. As mentioned previously, these have been divided pharmacologically into two broad classes:  $\alpha$  and  $\beta$ . With the exception of the intestinal tract,  $\alpha$ -adrenoceptor activation brings about contraction of smooth muscle (Ahlquist, 1981). Activation of  $\beta$ -adrenoceptors causes relaxation or inhibition of smooth muscle activity and also stimulation of the heart (Ahlquist, 1981). It is now clear that both these receptors can be further divided

into subgroups and each of these will now briefly be discussed.

There are two basic types of  $\alpha$ -adrenoceptor, designated  $\alpha_1$  and  $\alpha_2$ , which differ in their selectivity for certain agonists and antagonists.  $\alpha_1$ -Receptors are located postsynaptically and their activation leads to changes in cell calcium fluxes and phosphatidylinositol metabolism. Both pre- and postsynaptic  $\alpha_2$ -receptors have been described. The presynaptic receptors cause feedback inhibition of noradrenaline release from noradrenergic nerve terminals; the postsynaptic ones bring about a decrease in cyclic AMP through inhibition of adenylate cyclase. It is also worth noting that many tissues (and perhaps individual cells) contain both types of  $\alpha$ -receptor (Exton, 1982; Jakobs; Aktories and Shultz, 1981; Keppens, Vandenheede and DeWulf, 1977; Billah and Michell, 1979).

There are also two basic types of  $\beta$ -adrenoceptor:  $\beta_1$  and  $\beta_2$ . This division is again based on the selectivity of certain agonists and antagonists and was dependent upon the tissue response being examined. The receptors in the heart and adipose tissue were classified as  $\beta_1$ ; those found in the bronchi and vascular smooth muscle as  $\beta_2$  (Lands, Arnold; McAuliff, Laduena and Brown, 1967; Nahorski, 1981). Lelerc; Rouot, Velly and Schwartz (1981) considered that these subgroups were in fact non-existent and that the agonist/antagonist selectivity was due to differences in the surrounding membrane environment of the receptors and the physico-chemical nature of the drug used. For example, the



rate of loss of an agonist by uptake or non-receptor binding could be quite different between various tissues and generate an apparent selectivity. More recently, however, both types of receptor have been isolated and characterised.  $\beta_2$ -receptors are functionally active as a dimer,  $\beta_1$ -receptors as monomers, although there does appear to be some degree of similarity in their fine structure (Venter and Fraser, 1983). All  $\beta$ -adrenoceptors are thought to act through stimulation of a three component adenylate cyclase system (Levitizki and Helmreich, 1979). As with the  $\alpha$ -adrenoceptors, there is also evidence that both  $\beta$ -receptor subgroups may co-exist on the same tissue (Nahorski, 1981).

It is now generally accepted that there are at least two distinct types of dopamine receptor, classified as D-1 and D-2 (Kebabian and Calne, 1979). D-1 receptors are linked to the stimulation of adenylate cyclase; the prototype of which is found in the parathyroid gland (Brown, Carrol and Aurbach, 1977). D-2 receptors on the other hand, cause a decrease in, or have no effect on, the level of cyclic AMP production; the prototype of these receptors is found in the anterior and intermediate lobes of the pituitary, where identification was made by the binding of [ $^3\text{H}$ ]butyrophenones with high affinity. These receptors also exhibit high affinity (nanomolar) with dopamine agonists (Cote, Eskay, Frey, Grewe, Munemura, Stoof, Tsuruta and Kebabian, 1982; Onali, Schwartz and Costa, 1981; Leff and Creese, 1983). As mentioned previously dopamine receptors are found in several peripheral locations. This has resulted in confusion over the classification of the dopamine receptor subtypes. For

example, the D2-receptor found in the pituitary is thought to be equivalent to the D-2 receptor (designated by Golberg and Kohli, 1979) which mediates the inhibition of postganglionic sympathetic nerve fibres (Golberg and Kohli, 1983); but the D-1 receptor located in the parathyroids is not identical to the D-1 receptor (designated by Golberg and Kohli, 1979) which is associated with smooth muscle relaxation (Golberg and Kohli, 1983). Another area where confusion is rife is in the nigrostriatal system. Two distinct populations of [ $^3\text{H}$ ] butyrophenone binding receptors have been distinguished: those found on the cortico-striate terminals have a low agonist affinity and have been termed D-4 receptors (Sokoloff, Martres and Schwartz, 1980). However, Creese (1982) and Leff and Creese (1983) suggest that D-2 receptors have a low and high affinity binding state which is regulated by guanine nucleotides, and that the D-4 receptor represents the low affinity binding state. One more dopamine receptor subclass has been located in this brain system, although yet again its existence is being contended. These receptors show a nanomolar affinity for [ $^3\text{H}$ ] agonists but only a micromolar affinity for butyrophenones. Titeler, List and Seeman (1979) designated these as D-3 sites. Lesion experiments have demonstrated that these sites are found mainly on striatal neurones (Leff and Creese, 1983). As with the D-2 receptors, however, Leff and Creese (1983) believe that D-1 receptors also have low and high affinity agonist binding states, and that the D-3 site is in fact the high affinity state of the D-1 receptor. The only way for this confusion to be cleared up is for experimenters to standardize assay conditions, use

the same agonists and antagonists, and perhaps to concentrate on one animal species. Once one classification system is agreed upon it will become easier to interpret results and design further experiments.

In insects only three biogenic amine mediated neuronal responses have studied to any degree: (1) the effect of octopamine on locust extensor tibiae muscle; (2) the effect of dopamine on the salivary gland of the cockroach; and (3) the role of serotonin in salivary secretion of the blowfly. Each of these will now be briefly discussed.

It has been shown that the neurone DUMETi is octopaminergic and innervates the extensor tibiae muscle of the locust hindleg (see General Introduction). The octopamine, which is released from neurosecretory terminals has a neuromodulatory role, with receptors on both the muscle and the nerve terminals themselves (Hoyle, 1975; Hoyle, Colquhoun and Williams, 1980; Evans and O'Shea 1977 and 1978). Evans (1981) carried extensive agonist/ antagonist competition experiments on the octopamine response, and from the results concluded that there were three distinct types of octopamine receptor which mediated different aspects of the whole octopamine response. OCTOPAMINE-1 receptors are located on a specialized proximal bundle of muscle fibres where they modulate the myogenic rhythm of the muscle. OCTOPAMINE-2B receptors are located postsynaptically and cause an increase in the relaxation rate of the twitch tension which results from the activation of either the fast or slow motoneurones. Finally, OCTOPAMINE-2A receptors are

located presynaptically on the terminals of the slow motoneurone where they bring about an increase in both the spontaneous and the neurally evoked release of transmitter. This study also indicated that all classes of the octopamine receptor were pharmacologically similar to vertebrate  $\alpha$ -adrenoceptors. Recent work by Evans (1984a and b); however, has provided evidence that unlike  $\alpha$ -receptors the OCTOPAMINE-2 receptors mediate their action through stimulation of adenylate cyclase - the response mediated by vertebrate  $\beta$ -adrenoceptors. But if one looks at this in the light of recent findings on the adenylate cyclase system it is possible to account for this apparent discrepancy (discussed below).

Hormone-sensitive adenylate cyclase is composed of at least three distinct units: the catalytic subunit which produces the cyclic AMP; the specific hormone-binding receptors; and the guanine nucleotide regulatory subunits (Rodbell, Birnbaumer, Pohl and Krans, 1971; Rodbell, 1980; Lefkowitz and Hoffman, 1981; Exton, 1982). These regulatory subunits can either be stimulatory (as in the case of  $\beta$ -adrenoceptors) or inhibitory (as in the case of some  $\alpha$ -adrenoceptors). There are also other subunits that have no effect on cyclase activity (for example, those that bind  $\alpha$ -adrenoceptors in the liver). Since various hormones can have multiple effects within a cell, Rodbell (1980) suggested that the properties of the same receptor may be modified by interaction with different types of regulatory subunit. This certainly seems to occur between different cells since it has been reported that catecholamines (acting through

vertebrate  $\alpha$ -adrenoceptors) can both stimulate (Chan and Exton, 1977) and inhibit (Sabol and Nirenberg, 1979) cyclic AMP production, and also mediate effects unrelated to adenylate cyclase (Blackmore, Brumby, Marks and Exton, 1978). In addition, it has been found that hormone receptors that do not activate adenylate in intact preparations, can do so after cell homogenization (Rodbell, 1980). Perhaps homogenization causes the different regulatory subunits to be much more freely available. Hence the effect of a hormone on a cell may no longer only be determined by the type receptor involved. With the aid of GTP-regulatory proteins one receptor type could also mediate different responses, thereby increasing the number of possible processes that could be controlled by one hormone. Receptor association with different regulatory subunits could explain why insect OCTOPAMINE-2 receptors and vertebrate  $\alpha$ -adrenoceptors are similar pharmacologically but not physiologically.

As mentioned previously, there are several lines of evidence to support the role of dopamine as the transmitter at the cockroach salivary gland (see House, 1980, for a fairly extensive review). Stimulation of the salivary duct nerve or exogenously applied dopamine cause acinar cell hyperpolarization and fluid secretion by the gland. Initially it was thought that cyclic AMP was not involved in the response, but then Grewe and Kebebian (1982) showed that dopamine produced an increase in the cyclic AMP content of the acinar cells. More recently still, Gray, Ginsborg and House (1984) found that: (i) exogenously applied cyclic AMP caused a dose-dependent secretory response but had no effect



on the cell's membrane potential; and (ii) maintained responses to cyclic AMP and dopamine require the presence of extracellular calcium. Hence it appears that the stimulus-secretion coupling in this gland involves a second messenger system which requires both calcium and cyclic AMP to give the full response. There are, however, a few questions about the above study. First, theophylline (a phosphodiesterase inhibitor) did not enhance the dopamine response, although IBMX (another esterase inhibitor) produced dose-dependent fluid secretion. But when IBMX was applied in the presence of a low concentration of dopamine the secretory response was reduced, and not enhanced as would be expected. In addition, it was found that phentolamine not only reduced the dopamine mediated response but also the IBMX mediated increase which would be expected to be unaffected by a receptor antagonist. This implies that IBMX is acting as a partial agonist, but against this is the fact that IBMX does not cause acinar cell hyperpolarization. On the other hand it could be that phentolamine acts as an antagonist at other sites in the system. Finally, it was seen that the diterpene forskolin, which directly stimulates the catalytic subunit of the adenylate cyclase system (Seamon and Daly, 1983), had a very limited stimulatory effect on the gland. More work is required on this preparation to adequately explain these results, although in support of the suggestion of two second messengers are the results of studies on blowfly salivary gland (see below).

The control of salivary gland secretion from the blow-fly is quite different from that of the cockroach. The gland is un-

innervated, the secretion being mediated by a substance released from neurosecretory axons in the haemolymph (see review by House, 1980). It is believed that serotonin is the unidentified neurohormone since it is the most potent physiological stimulant yet found (Berridge, 1972). Prince, Berridge and Rasmussen (1972) provided direct evidence that serotonin increases the cyclic-AMP content of the glands. But it has also been shown that cyclic-AMP alone does not give the complete serotonin response (Berridge and Prince, 1972); extracellular calcium is also required (Prince et al, 1972). In the light of this Berridge (1979) proposed that serotonin mediates its response through two distinct receptors: one linked to the adenylate cyclase system; and the other to calcium channels.

Other putative adenylate cyclase-linked biogenic amine receptors have been isolated from insect central nervous system (Nathanson and Greengard, 1973 and 1974; Harmar and Horn, 1977; Bodnaryk, 1982). However nothing is known about their possible function since all of these experiments were carried out on homogenized preparations. It was mentioned above that homogenization could show up an adenylate linked response which was not present in intact preparations. Recently it has been shown that some of the properties of vertebrate  $\beta$ -adrenoceptors adenylate cyclase system also alter with homogenization (Porzig, 1982). Hence what is required with insects is detailed experiments on intact preparations, both to classify the receptors more clearly and to discern their function.

Since it had proved very difficult to identify any of the

dopamine-containing neurones morphologically it was decided to discover if any of the cells in the prothoracic ganglion responded to dopamine. Once a cell was located the response could be characterized to a certain degree and the cell indentified. Although all the ventral nerve cord ganglia have biogenic amine-containing neurones it is hoped that future attempts to fill the prothroracic medial pair described in Chapter 1 will be successful. This could then be followed by identifying some of the cells with which it has connections. Hence, by focusing on this one ganglion it should be eventually possible to correlate the physiological and morphological results.



MATERIALS AND METHODS

The experimental apparatus was very similar to that used for the GABA/glutamate experiments (Chapter 2, section I). The preparation was set up in the same way as in the intracellular injection experiments involving the prothoracic ganglion (Chapter 1, section II). The alterations and additions to the apparatus and procedures were as follows.

The aim of these experiments was to identify cells in the prothoracic ganglion that have receptors for dopamine and then to characterize them pharmacologically. Initially cells on both the dorsal and ventral surface were impaled and the response to pressure ejecting dopamine onto their surface ascertained. Once a responsive cell was obtained the experiments that followed concentrated on identifying the cell and characterizing the response.

In these experiments current was passed through the microelectrode using the Grass S44 stimulator to which it was connected via the amplifier /bridge circuit. The input to the oscilloscope was also taken to a Gould Mark 220 pen recorder. In addition, this was connected to a digital voltmeter which allowed the cell's membrane potential to be constantly monitored.

The recording microelectrodes and holders were filled with 2M potassium acetate. Their resistance was usually 10-20 Mohms. The picospritzing microelectrodes were filled with one of the following: 0.1M dopamine, 0.1M noradrenaline, 0.1M octopamine, 0.1M  $\gamma$ -aminobutyric acid, 0.1M acetyl-

choline, 0.1M 5-hydroxytryptamine (all supplied by Sigma). Each solution was made up in saline and prepared daily, just before it was required. Dopamine, in particular, is very susceptible to oxidation and so for this a fresh solution was used for every preparation.

\* Propranolol (Sigma) and phentolamine (Ciba) were made up in distilled water to a 10 mM stock solution and stored frozen; picrotoxin (Sigma) - a stock solution of 1.7 mM in saline was used; 3-isobutly-1-methylxanthine (IBMX; Sigma) - a stock solution of 3 mM in saline was stored frozen;  $N^6, O^{2'}$ -dibutyryl adenosine 3':5'-cyclic monophosphoric acid (dBcAMP; Sigma) - a 10 mM stock solution in saline was used; forskolin (Cal-Biochem) - 1 mM and 50 mM stock solutions in ethanol were used. Solutions that were not stored frozen were made up fresh just before the start of the experiment. The drugs were added directly to the experimental bath using a Gilson pipette.

After several experiments it was found that one particular neurone responded to dopamine and could be consistently impaled in different preparations. In some of the following experiments, therefore, the dopamine response was first confirmed and the recording microelectrode was removed and the cell was reimpaled with a microelectrode filled with 100 mM hexaminecobaltic chloride. Microinjection of the cobalt was carried out using current passed directly into the microelectrode from the Grass stimulator via a 100Mohm resistor (see Chapter 1, section III). The injection was

continued for 25-45 min. Once it was complete the preparation was removed from the bath and left in saline for about 15 mins. The cobalt was then precipitated out as cobalt sulphide. Four of the preparations were dehydrated in alcohol, cleared and stored in creosote (see Chapter 1, section II). The remaining three preparations were intensified (see Chapter 1, section III and Table 1.8)

Stereo photographs of the intensified preparations were taken as described previously (Chapter 1, section III).

#### Drawings of the Intensified Cell

The preparation was mounted in creosote on glass backed aluminium slide. An image of the ganglion was projected onto a piece of paper using a Gillett and Sibert microscope fitted with a projecting prism attachment. Starting on one surface of the preparation, the image was focussed sharply and drawn. This was repeated for different planes through the ganglion. This enabled an accurate picture of the cell to be drawn. Although this did give a detailed picture of the branching pattern of the cell, three dimensional information was lost. When taken along with the stereo photographs, however, a good overall picture of cell position and anatomy is obtained.

Very bright light had to be used to show up the fine branching of the cell and allow it to be drawn. This light, however, caused the tissue to turn brown with time, which in turn caused the fine branching to gradually become indistinguishable from the background. It was essential, therefore, to draw the preparations as quickly as possible.

Also, since this deterioration in visability was irreversable, the drawing of the cell was carried out after the photographic records were complete.

RESULTS

A large (about 70  $\mu$ m) pair of neurones situated on the ventral surface of the ganglion near the midline were found to consistently give a depolarization in response to pressure ejected and bath applied dopamine. After injection of hexaminecobaltic chloride and silver intensification of the preparation it was found that the pair corresponded to the common inhibitory neurones, D3 (Pearson and Fourtner, 1973; Iles, 1976 ; Figs. 2.16 and 2.17). D3 sends a process dorsally into the neuropile. This very quickly splits into two branches; one continues dorsally, the finner branches of which are seen in Fig. 2.17 (they appear to be coming directly from the cell body). The other process runs laterally and divides to send branches directly into nerves 3, 4 and 6. A branch is sent into nerve 5 via the small nerve anasomosis that connects nerves 4 and 5. The branches in nerves 6 and 3 (not shown) further divide into roots A and B of these nerves. A fine branch innervates the anterior lateral edge of the ganglion but most of the branching occurs in the neuropile caudal to the main lateral axon. The branches in the peripheral nerves innervate a large number of functionally different muscles in the coxa (Pearson and Iles, 1971).

After impaling D3 it was often possible to record small action potentials (about 5-10 mV). Pearson and Fourtner (1973) also found this and showed that these potentials could be correlated with activity in the peripheral nerves. They are thought to be due to electrotonic spread from electrically active regions of the axon.

All the results which will now be described were obtained from recordings made in prothoracic common inhibitory neurone (D3).

Pressure ejected dopamine pulses produce long lasting (40-60 sec) depolarizations in D3 (Fig. 2.18a). There was also a small decrease in cell membrane resistance since the height of the stimulating pulses decreased slightly (from 10 mV to 9 mV in Fig. 2.18a). This decrease was usually small and was even more difficult to see when the cell was firing the small action potentials.

When a final concentration of 0.1 mM dopamine was applied to the bath the cell rapidly depolarized (Fig. 2.19b, c and d); 6.5 mV and 11 mV after 40 and 80 sec respectively. On prolonged exposure (over one hour) the cell remained depolarized, washing had little effect. The membrane resistance was only slightly decreased. It is hard to tell if the dopamine had an effect on the frequency of the small potentials independent of the increase due to the depolarization itself. On addition of 0.1  $\mu$ M dopamine (final concentration) to the bath of a preparation whose excitatory potential firing rate was low to begin with it was seen that there was a significant increase in this although there was negligible change in the membrane potential over 30 min (about 1 mV; Fig. 2.19 a-e). After washing the <sup>action</sup>potential frequency was greatly decreased and it was seen that the <sup>action</sup>potentials tended to come in bursts (Fig. 2.19f). This bursting was also sometimes observed when the dopamine was pressure ejected onto the cells, especially those that were

not receiving regular hyperpolarizing pulses (Fig. 2.19g). The depolarization seen in this example was very small (only 1 mV). This was probably caused by the dopamine having limited access to the cell surface. The D3 cells appear to be fairly deep lying since they were usually difficult to see before the surrounding glial cells were cleared away; cell 28 on the other hand could normally be spotted immediately. Normally no response was obtained with pressure ejected dopamine if the glial cells were left untouched. In the preparations where larger depolarizations were seen, the action potential frequency increased in response to this and any bursting of the potentials would have tended to become obscured.

It was found that dopamine and noradrénaline had about equal potency in depolarizing the cell; octopamine was much less effective (Fig 2.20 a-d); and serotonin had no effect.

Phentolamine (final concentration 25  $\mu$ M) was added to <sup>the</sup> bath and the dopamine response monitored. The response was maximally reduced after 4 min (Fig. 2.21a). After washing it returned to normal. The effect of ( $\pm$ ) propranolol (final concentration 25  $\mu$ M) was determined on the same preparation (Fig. 2.21b). It was more effective at reducing the response which again was returned to normal on washing. Higher concentrations of both drugs increased the degree of blockade but then their effect was difficult to reverse. Neither drug was able to cause 100% inhibition when used on its own at concentrations up to 0.1 mM.

On some preparations both phentolamine and propranolol were



added together (Fig. 2.22). It was found that a 1:1 mixture of the two drugs was more effective at inhibiting the response than an equivalent concentration of either alone. It may be seen that the response was completely abolished by the mixture (Fig. 2.22a). In each case the response was restored after washing the preparation.

It was decided to check whether the two drugs had any effect on other types of response. It was found that prolonged exposure (up to an hour) to either drug had no effect on the hyperpolarizing response induced by GABA. However, it was found that both drugs significantly decreased the depolarizing response produced by acetylcholine (Fig. 2.23) at concentrations equivalent to those used to inhibit the dopamine response.

Several experiments were carried out to determine whether or not dopamine acted through cyclic AMP. The phosphodiesterase inhibitor IBMX (final concentration 0.25 mM), which might be expected to potentiate the dopamine response, either had no effect on the response (Fig. 2.24a) or in some preparations actually decreased it (Fig. 2.24b).

Dibutyryl cyclic AMP (final concentration 1 mM), a membrane soluble analogue of cyclic AMP, was added to the preparation in the presence of 0.1 mM IBMX. It produced no effect on its own and did not affect the dopamine response (Fig. 2.25a and b) except on prolonged exposure when it produced a slight inhibition (Fig. 2.25d). When dopamine was added to the bath (0.1 mM) the cell depolarized as before and rapidly ceased to respond to pressure ejected dopamine (Fig. 2.25d) showing



that the receptors were already maximally stimulated.

Addition of 50  $\mu$ M (final concentration) forskolin had no effect on the dopamine response, even after 30 min although the drug did increase the frequency of the small action potentials (Fig. 2.26a and b). At 35 min (Fig. 2.26a) and 19 min (Fig. 2.26b); 0.1 mM (final concentration) dopamine was added to the bath. Again the cells depolarized and no longer responded to pressure ejected dopamine (Fig. 2.26a and b; last traces).

Figure 2.16 Diagram of a cockroach prothoracic ganglion (dorsal view) showing the axonal projections and dendritic field of the common inhibitory neurone D3. The neurone had been injected with hexamminecobaltic chloride and then silver intensified. The cell body and initial segment of the axon are outlined with a dotted line. Calibration 100  $\mu$ m.

**ANT**

**POST**

**N3**

**N5**

**N4**

**N6**

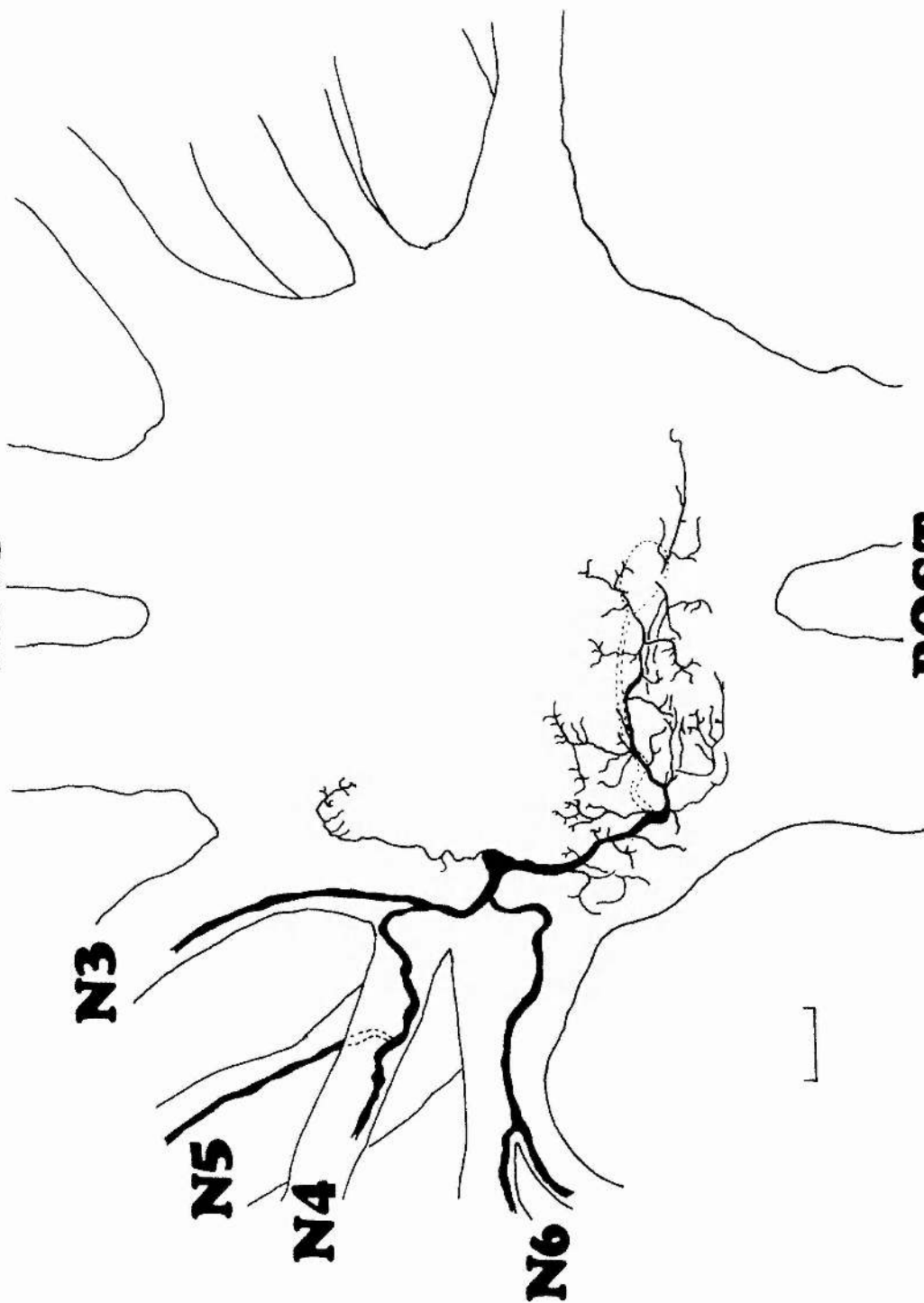


Figure 2.17 Photograph of a cockroach prothoracic ganglion after D3 had been injected with hexamminecobaltic chloride and then silver intensified. The ganglion is ventral side up and so the cell body and its laterally projecting axon are clearly seen. Only two (N4 and N6) of the peripherally projecting axons are visible in this focal plane. Calibration 100  $\mu$ M.



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Figure 2.18 (a)-(d) The effect of pressure ejected and bath applied dopamine upon the neuronal membrane of D3

(a) Pressure ejected dopamine (200 ms 15 psi) produced a 40 sec duration depolarization at both -59 mV and -69 mV. The cell was stimulated with 500 ms duration, 10 mV hyperpolarizing pulses throughout the experiment - the pulses appear larger due to the presence of the small action potentials which are seen more clearly in (b)-(d). It may be seen that the response is smaller at the less negative potential. During the response the height of the stimulating pulses decreased slightly (only clear in the original trace), indicating that the cell's membrane conductance had slightly increased.

(b)-(d) 0.1 mM dopamine was applied to the bath and resulted in rapid depolarization of the cell; 6.5 mV and 11 mV after 40 and 80 sec respectively.

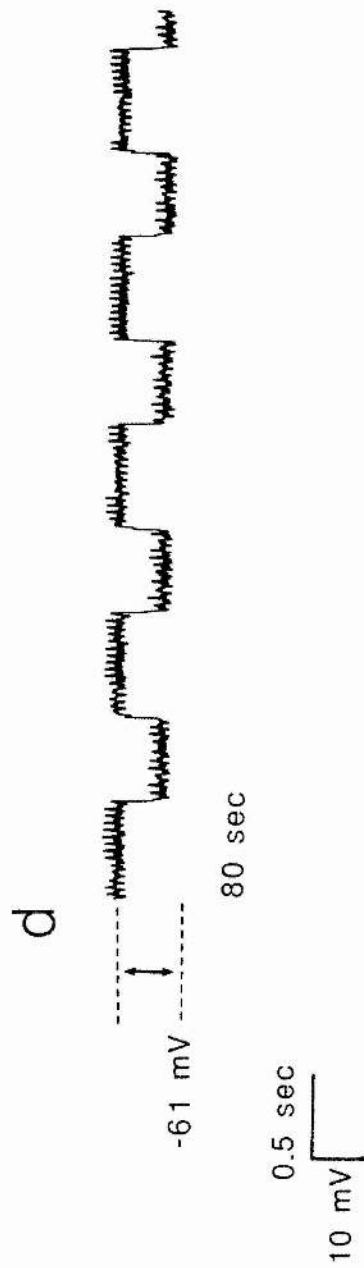
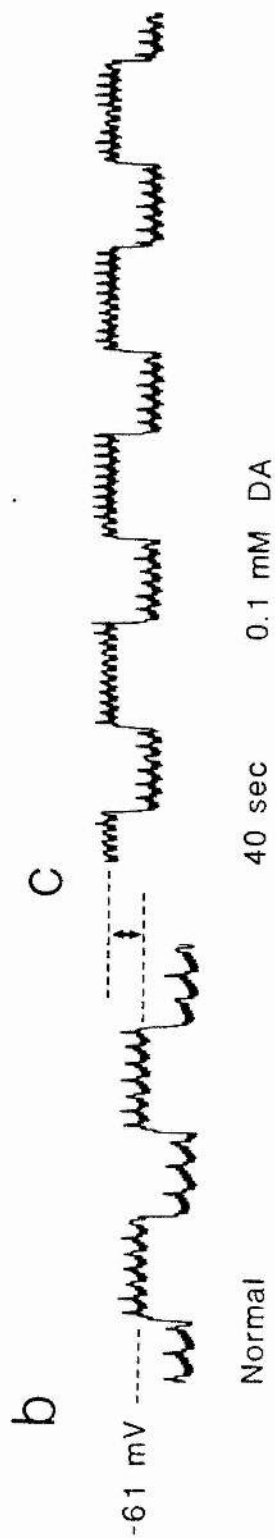


Figure 2.19 (a)-(g) The effect of bath applied and pressure ejected dopamine upon the neuronal membrane of D3.

(a)-(f) Bath application of 0.1  $\mu$ M dopamine increased the frequency of the small action potentials; the membrane potential only decreased by 1 mV over 30 min. After washing it may be seen that the small potentials tended to come in bursts (f). The membrane potential was -62 mV

(g) Pressure ejection of dopamine (150 ms 15 psi) onto non-pulsed cells produced a depolarization (only 1 mV in this case) and sometimes also bursting of the small action potentials. Membrane potential was -59 mV





Normal



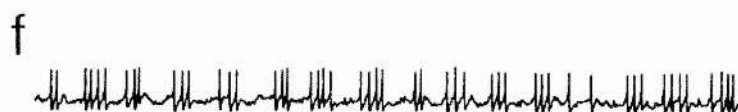
6 min DA 0.1  $\mu$ M



14 min



30 min



4 min Wash



Figure 2.20 (a)-(d) Comparison of the effect of pressure ejected dopamine, noradrenaline and octopamine upon the neuronal membrane of D3.

(a) and (b) show that dopamine and noradrenaline produce an equivalent response. Octopamine is much less effective (c), even at higher concentrations (d). The preparation was stimulated as in Fig. 2.18a; the membrane potential was -63 mV.

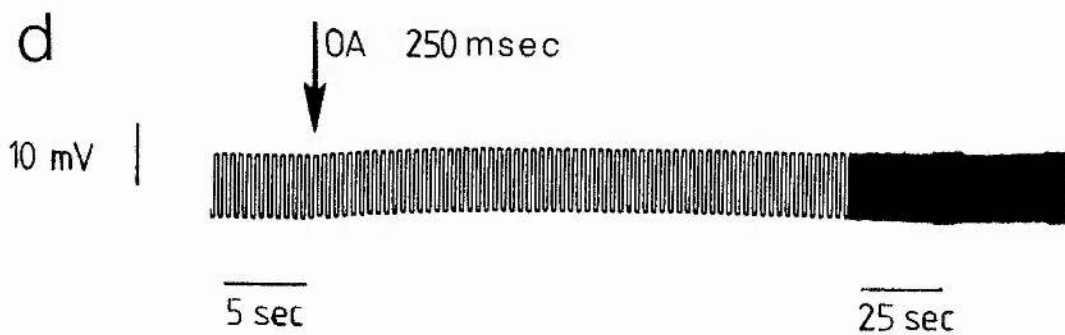
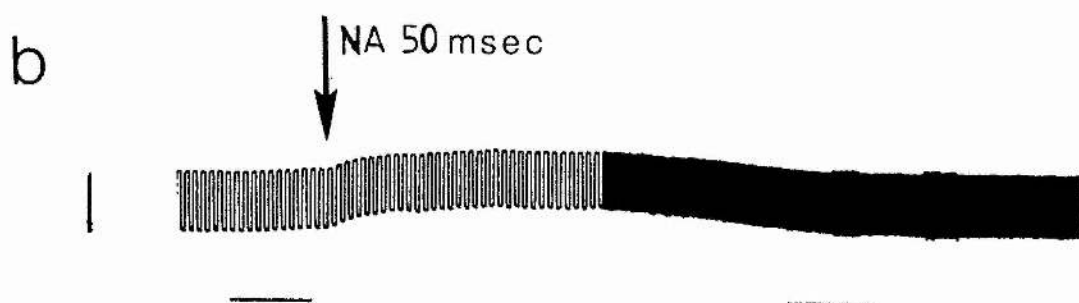
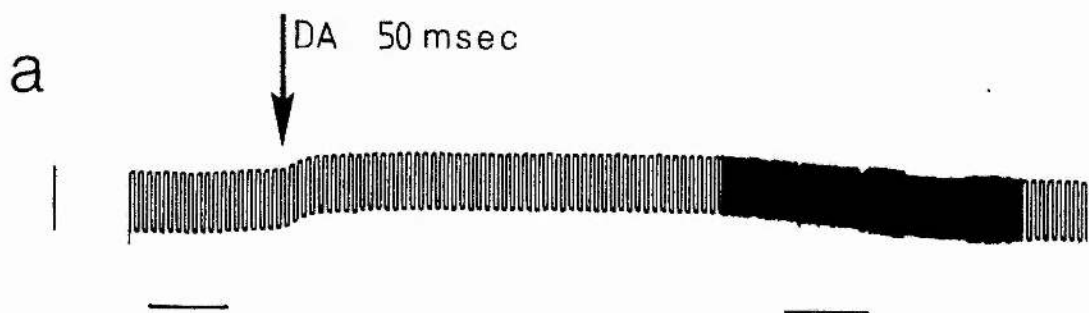


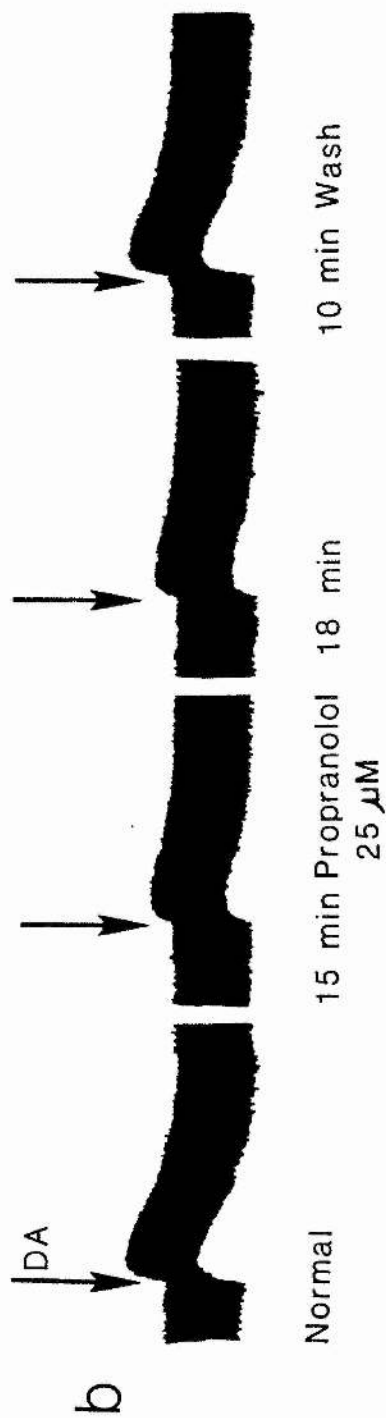
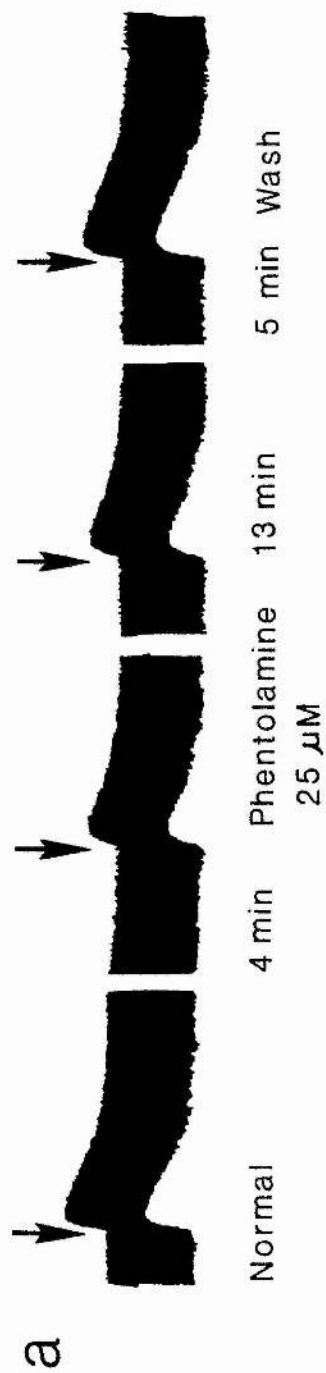
Figure 2.21 (a) and (b) The effect of bath applied phentolamine and propranolol upon the response to pressure ejected dopamine (200 ms, 15 psi).

(a) Phentolamine (25  $\mu$ M) produced a reversible decrease in the response.

(b) Propranolol also produced a reversible decrease.

In both cases the preparation was not washed until the maximal decrease was obtained. It may be seen that propranolol is a more effective antagonist, although neither drug completely abolished the response at the concentration used.

The preparation was stimulated as in Fig 2.18a; the membrane potential was -61 mV.




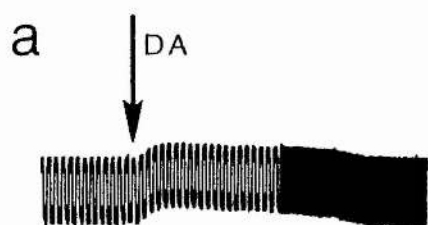
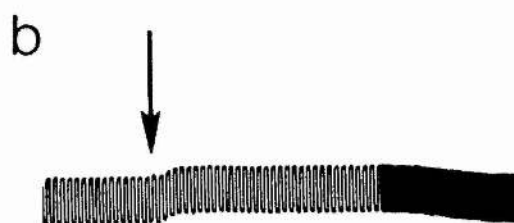
10mV  25 sec

Figure 2.22 (a)-(h) The effect of a combined bath application of phentolamine and propranolol upon the response to pressure ejected dopamine (50 ms; 15 psi). (a)/(b) and (c)/(d) show that 25  $\mu$ M propranolol or phentolamine alone reduces but does not totally abolish the response. When added together (1:1), at a combined concentration of 25  $\mu$ M, it may be seen that the response completely disappeared (g), but was returned to normal after washing (h). This suggests that there could be at least two types of dopamine receptor, with each drug acting preferentially at one of them.

The preparation was stimulated as in Fig. 2.18a; the membrane potential was -62 mV.



Normal



15 min Propranolol 25  $\mu$ M



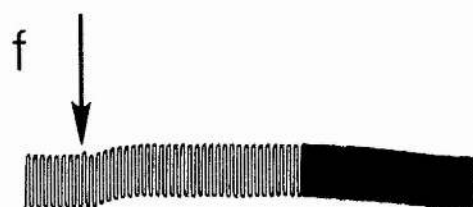
20 min Wash



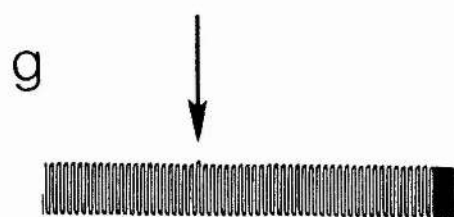
20 min Phentolamine 25  $\mu$ M



25 min Wash



9 min Phentolamine 12.5  $\mu$ M  
0 min Propranolol 12.5  $\mu$ M



18 min Phentolamine  
9 min Propranolol



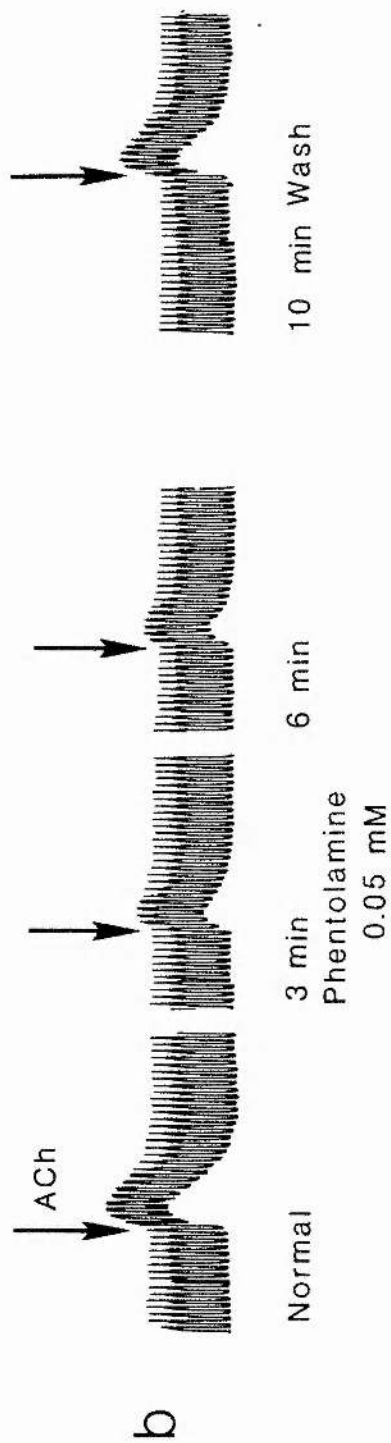
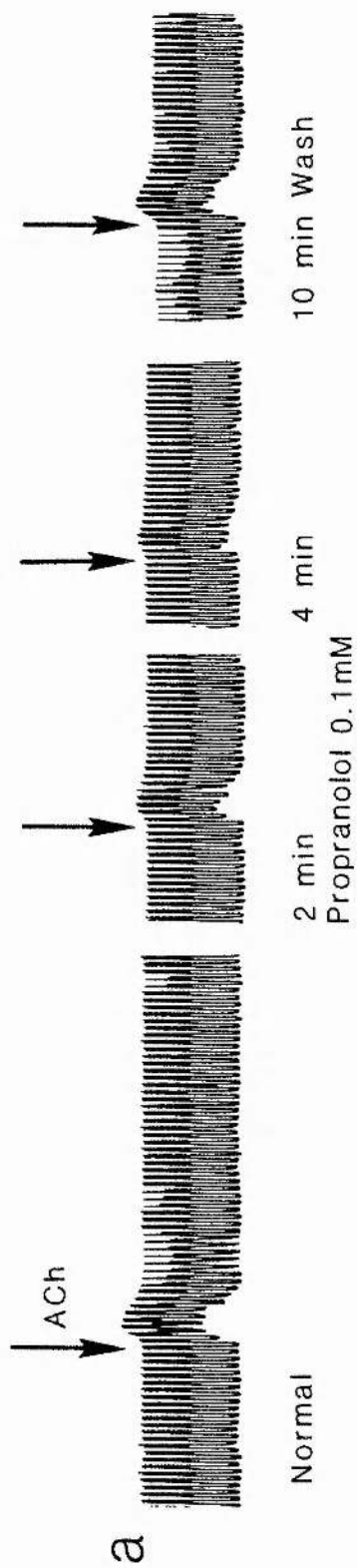
25 min Wash


10 mV

Figure 2.23 (a) and (b) The effect of phentolamine and propranolol upon the response to pressure ejected acetylcholine. It may be seen that both propranolol (0.1 mM) and phentolamine (0.05 mM) reversibly decreased the response. This suggests that either the two drugs can act at both acetylcholine and dopamine receptors, or they act at the ion channels controlled by the receptors (these would have to be the same for both receptors).

The preparations were stimulated as in Fig. 2.18a; the membrane potentials were -62 mV and -59 mV respectively.





10 mV  5 sec

7

Figure 2.24 (a) and (b) The effect of bath applied IBMX (0.25 mM) upon the response to pressure ejected dopamine (50 ms; 15 psi). It may be seen that the the IBMX either had no effect (a) or produced a reversible decrease (b) in the response. The neurones were stimulated as in Fig. 2.18a; the membrane potentials were -59 mV and -62 mV respectively.

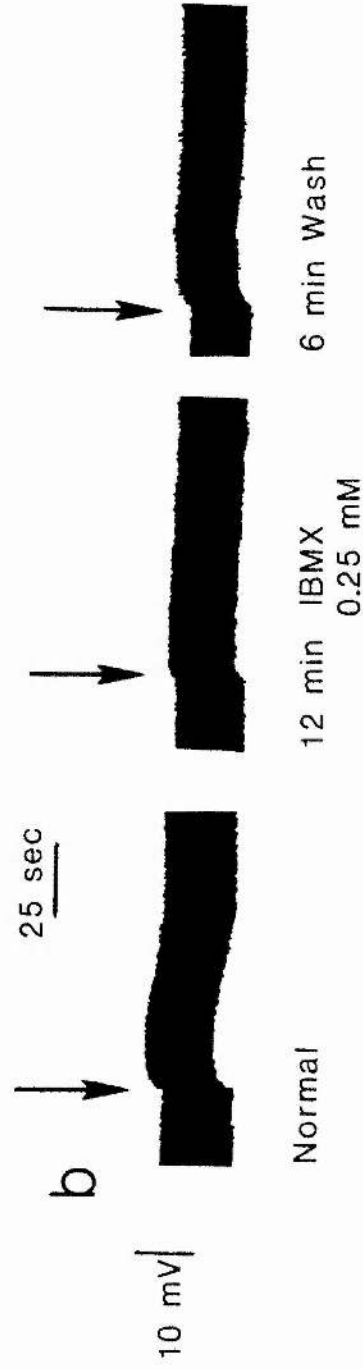
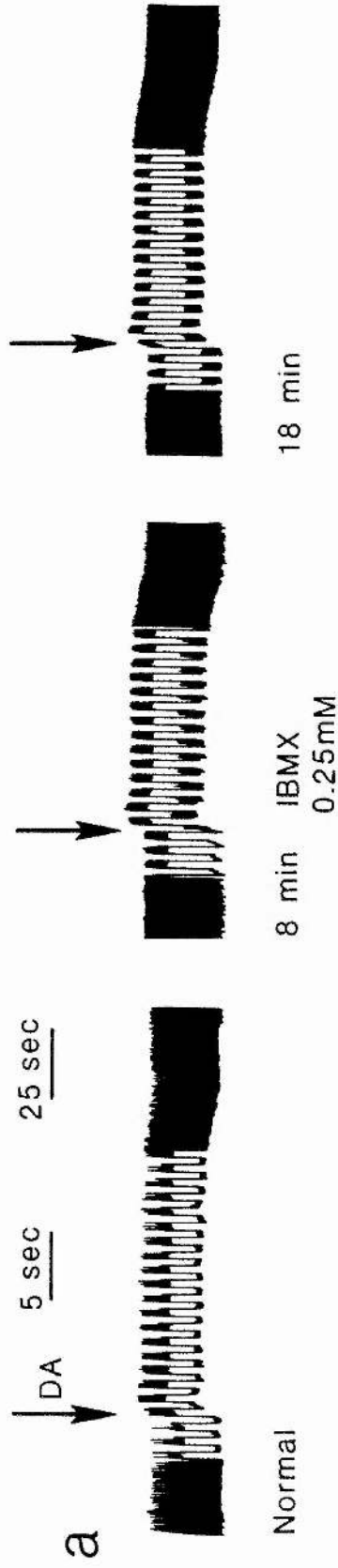


Figure 2.25 The effect of bath applied dibutyryl cyclic AMP upon the response to pressure ejected dopamine (100 ms, 15 psi). The cyclic AMP analogue was added in the presence of 0.1 mM IBMX. It may be seen that after 19 min there was a slight decrease in the dopamine response (c). At 23 min 0.1 mM dopamine was added to the bath (d). The cell depolarized (as in Fig. 2.18) and no longer responded to pressure ejected dopamine. The cell was stimulated as in Fig. 2.18a; the membrane potential was -63 mV.

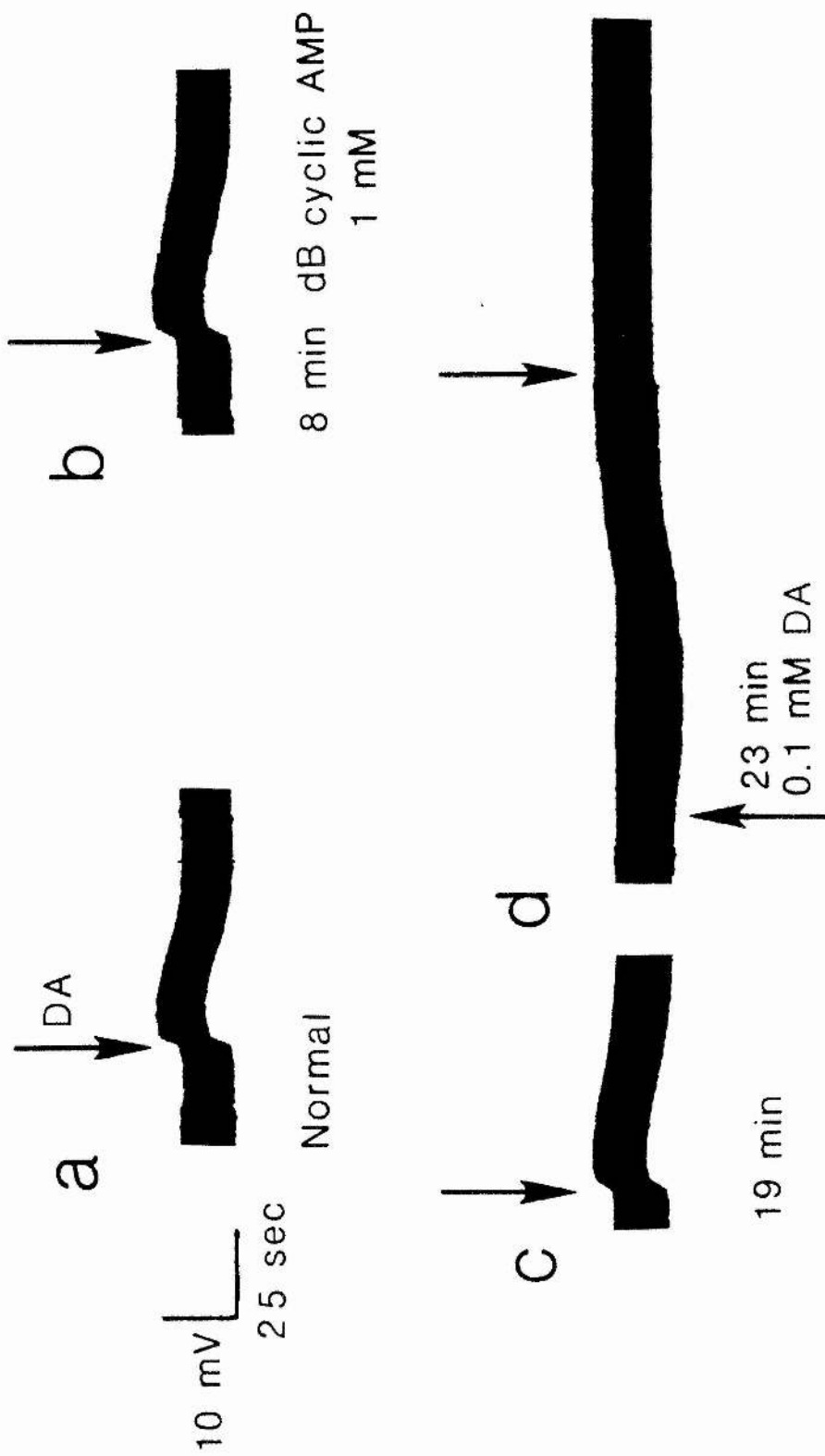
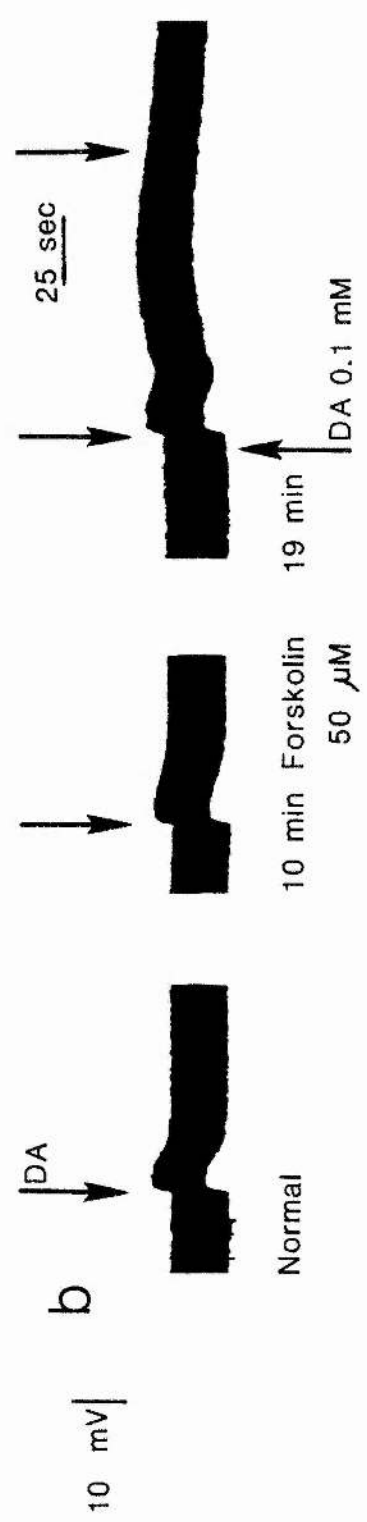
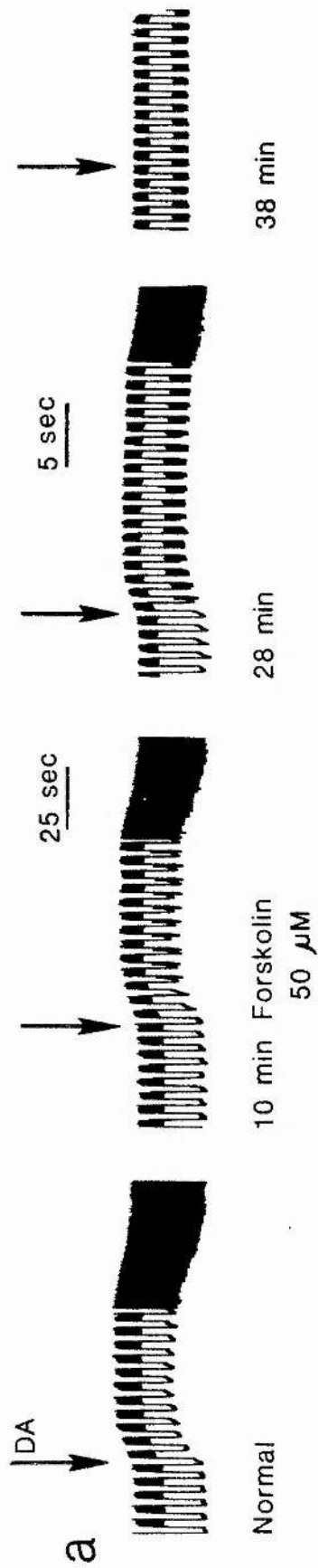


Figure 2.26 (a) and (b) The effect of bath applied forskolin (50  $\mu$ M) upon the response to pressure ejected dopamine.

(a) It may be seen that forskolin has little effect on the dopamine response the height of the response (at the more negative potential) in the 3rd trace is partly obscured by the small action potentials. At 35 min 0.1 mM dopamine was added to the bath. The cell depolarized (compare levels of trace 3 and 4) and no longer responded to pressure ejected dopamine (trace 4). It may also be seen that the forskolin increased the frequency of the small potentials (compare more negative potentials in traces 1-4).

(b) Another example of the lack of effect of forskolin on the dopamine response. As before, the addition of 0.1 mM dopamine to the bath maximally stimulates the receptors and hence prevents any response to further bursts of pressure ejected dopamine (trace 3).

The cells were stimulated as in Fig. 2.18a; the membrane potentials were -58 mV and -60 mV respectively.



DISCUSSION

Only a few electrophysiological experiments have been carried out to determine the effect of catecholamines on insect central neurones. In general though, dopamine, noradrenaline and adrenaline have been found to be excitatory (e.g. Kerkut et al., 1969b; Walker, James, Roberts and Kerkut, 1980; see also the General Introduction), where the recordings have been made from either ganglionic connectives (Twarog and Roeder 1957; Gahery and Boistel, 1965) or unidentified cockroach DUM neurones (Kerkut et al., 1969b; Roberts and Walker, 1981).

In this study it has been shown that both bath applied and pressure ejected dopamine depolarizes and excites the common inhibitor motoneurone D3 of the cockroach prothoracic ganglion. This excitation may have been brought about by excitatory connections from other neurones but such a possibility is unlikely. As insect neurones do not have synapses on their cell bodies, post-synaptic excitation would have electrotonically spread from synaptic regions of the cell resulting in a high degree of signal attenuation. The size of the somatic depolarization recorded was often about 10 mV, i.e. about an order of magnitude greater than a plausible synaptic potential. In addition, the pressure ejecting microelectrodes were placed directly over the cell body; if they were held too close, desensitization of the response occurred, whereas if raised too far away, no response was obtained. It is unlikely that this result would be obtained through stimulation of other neurones and hence



the cell body of neurone D3 must have receptors that respond to dopamine. The next question that must be considered is whether or not the responses are a result of action on dopamine specific receptors.

#### Characterization of the Dopamine-Sensitive Receptors

Both GABA and glutamate produce hyperpolarizing responses that are blocked by picrotoxin but not by phentolamine or propranolol. Hence dopamine does not act on receptors for these amino acids.

Acetylcholine, however, does give a depolarization which is reversibly inhibited by both phentolamine and propranolol (Fig. 2.23). The response time is much shorter (5-10 sec for ACh compared with 30-60 sec for dopamine; compare Fig. 2.23 with any Fig. showing a dopamine response) but it is known that acetylcholine is rapidly removed by the action of acetylcholinesterase (see review by Sattelle, 1980). Hence it is possible that dopamine may be acting at the cell's cholinergic receptors, which in the absence of a specific inactivating mechanism produced a longer lasting response. The depolarization produced by iontophoresis of acetylcholine onto the soma of metathoracic cell 28 has been well studied (David and Pitman, 1979 and 82; David and Sattelle, 1984). This response was also readily obtained in the present work. Pressure ejected dopamine, however, produced no response in cell 28. Also in one experiment the response to pressure ejected acetylcholine on D3 was monitored after the addition of 0.2  $\mu\text{M}$  dopamine (final concentration) to the bath. As seen before with 0.1  $\mu\text{M}$

dopamine (Fig. 2.19a-e) the frequency of the action potentials increased, thus indicating that dopamine was acting on the preparation. However, after 30 min the maximal acetylcholine response had only decreased from 10 to 9 mV. These results suggest that the receptors for dopamine are distinct from those of acetylcholine although it would be necessary to perform further experiments to prove the distinction, the following being crucial:

- a) To determine the reversal potential for both responses in D3. Different reversal potentials would indicate two distinct populations of ligand activated channels.
- b) Bath apply dopamine or acetylcholine and monitor the response to both pressure ejected dopamine and acetylcholine. If the receptors are different then only the pressure ejected acetylcholine response should be significantly decreased when acetylcholine is present in the bath, whereas only the pressure ejected dopamine response should be significantly affected when dopamine is added to the bath.
- c) To ascertain the effects of cholinergic antagonists (see General Introduction) and other possible dopaminergic antagonists (see later in Discussion) on both responses. If the order of potency of the drugs is different for each response then it is likely the receptors are different.

As discussed in the General Introduction, the insect nervous system contains (as well as dopamine) the biogenic amines noradrenaline, serotonin and octopamine. Any any of these may have receptors on neurone D3 at which dopamine acts. However, several workers have obtained evidence for distinct

dopamine, octopamine and serotonin receptors in the cockroach nervous system. This is summarised in the following paragraph.

Nathanson and Greengard (1973) identified a dopaminergic, an octopaminergic and a serotonergic dependent adenylate cyclase in homogenates of cockroach thoracic ganglia. For both dopamine and octopamine dependent enzymes the  $\alpha$ -adrenergic blocker, phentolamine, was much more effective at inhibiting the activation produced than the  $\beta$ -adrenergic blocker, propranolol. Neither of these drugs were effective at blocking the serotonin-dependent stimulation. Nathanson and Greengard (1973) also found noradrenaline stimulated activity as well but only at higher concentrations. By carrying out experiments in which two amines were simultaneously added, each at concentrations which maximally stimulated the adenylate cyclase, they were able to show that noradrenaline had no specific receptor, and was simply exerting its effect through the octopamine and dopamine receptors. Harmar and Horn (1977) also detected a dopamine sensitive adenylate cyclase from cockroach brain, which was potently inhibited by  $\alpha$ -flupenthixol. As was mentioned above (see Introduction), dopamine is considered to be a transmitter at the neuroglandular junctions of cockroach salivary glands (House, 1980). Bowser-Riley, House and Smith, (1978) found that phentolamine competitively inhibited the electrical and the secretory response of isolated cockroach salivary glands to both nerve stimulation and bath application of agonists.  $\alpha$ -Flupenthixol also inhibited the responses, but this time the results suggested

that it acts in a non-competitive manner; propranolol was ineffective (House and Smith, 1980; Ginsborg and House, 1980). Grewe and Keababian (1982) working on the same cockroach species (Nauphoeta cinerea) reported that dopamine increased cyclic AMP production in the salivary glands but phentolamine only partially antagonized the dopamine-mediated increase and propranolol was equally effective.

In the present study it was found that noradrenaline and dopamine were equally effective at depolarizing D3. Since dopamine is present in much higher concentrations in the insect central nervous system (ten fold greater), it is more likely that the natural ligand is dopamine. Agonist/antagonist studies have been carried out in various vertebrate and invertebrate species (for example molluscs) but trans-species comparisons are of limited value. It has already been shown that both phentolamine and propranolol partially inhibit the dopamine response in D3, but these drugs have no effect on vertebrate dopamine responses. Of course, it is possible that D3 could have distinct noradrenaline and dopamine receptors at which either could act. It was in fact shown that both drugs together were more effective at inhibiting the dopamine response than an equivalent concentration of one alone. In addition, the receptors reported here may be different from the dopamine ones found at the cockroach salivary gland (Bowser-Riley et al., 1978; Ginsborg and House, 1980) or in brain homogenates, and also from the octopaminergic receptors found at the locust neuromuscular junction (Evans, 1981) or

in brain homogenates (Nathanson and Greengard, 1973), where in each case phentolamine was a much better inhibitor than propranolol. On the other hand, the results of Grewe and Keababian (1982) suggest that the dopamine sensitive receptors on D3 and on salivary gland cells may be pharmacologically similar, although the effect of cis-flupenthane (which these authors found to be a powerful dopamine antagonist) on the dopamine response of D3 is not yet known and no mention was made in their study of whether or not the antagonism by phentolamine and propranolol at the salivary gland was additive.

Octopamine produced a much smaller response than dopamine on neurone D3, even at higher concentrations. It is possible that octopamine is the natural ligand for the receptor activated by dopamine and that its more rapid inactivation results in a smaller response. On the other hand, in Chapter 1, section 1, it was shown that each ventral nerve cord ganglion contains a very extensive network of catecholamine-containing fibres, which are most probably dopaminergic. Hence one would expect any dopamine inactivating mechanism to be just as extensive, although it is unknown if such a mechanism exists in the vicinity of the cell body of D3. Recent work by Sombati and Hoyle (1984 a and b) indicates that centrally released octopamine may have a role in the modulation and/or generation of specific behaviours in the locust. The duration of the octopamine responses were long-lived, lasting tens of seconds beyond the duration of octopamine application. These authors suggested that this was due to a lack of specific enzymes for octopamine

inactivation, with the amine probably being taken up by surrounding glial cells and degraded within them.

To discover whether or not dopamine and octopamine are acting at the same receptors it is necessary to determine the potency of various dopaminergic and octopaminergic antagonists (listed below) on both responses. If the order of effectiveness is found to be different, then it is likely that the amines act at different receptors. If the order is found to be the same, then only one receptor is involved. Some idea of which amine is the natural ligand may be gained by determining whether the most effective antagonists are classed as dopaminergic or octopaminergic. Further evidence could be obtained by determining which class of agonist (listed below) is the most potent at producing the depolarizing response.

Serotonin had no effect on the cell and it was concluded that serotonin receptors were not involved in the dopamine response.

A fundamental question is the suitability of using either phentolamine or propranolol as antagonists in the cockroach nervous system. It was stated in the results that the acetylcholine response of D3 was also reversibly blocked by these two drugs. The possible explanations which arise are as follows: (i) the drugs can act at both cholinergic and catecholaminergic receptors; (ii) they act in a non-competitive fashion at a site distinct from the receptors but still work by inhibiting agonist binding; (iii) they



bind to and block the receptor-coupled ionophores; (iv) they inhibit the coupling between receptors and ionophores. Although it is not known which of the above actually apply, the results do emphasize that drugs that are specific in one preparation may not be in others and hence show the importance of checking the effect of a drug on various responses when it is being used in an untried preparation.

Taken together these results suggest that there are octopamine and/or catecholamine receptors present on the cell body of D3. During the present series of experiments the receptors were further characterized using dopamine since the response was fairly large and consistent.

#### Possible Drugs for Receptor Characterization

From the results of experiments in both invertebrate and vertebrate preparations, suggestions of drugs that may be useful in characterizing the receptors on D3 are given in the following three paragraphs. Experiments using these drugs may help determine the nature of the endogenous ligand of the D3 receptors and also provide information about (1), the conformation of the ligand when bound to the receptor and (2), the chemical groups required for receptor activation.

Possible dopamine agonists to test include epinine, A-6,7-DTN (2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene) and apomorphine. Harmar and Horn (1977) found that both epinine and A-6,7-DTN were comparable in potency to dopamine in activating adenylate cyclase in cockroach brain homogenates:

both have also been found to be potent agonists in vertebrate systems (Kebabian and Calne, 1979). In fact A-6,7-DTN is active on all dopamine systems so far examined (Woodruff, 1982), even being equipotent with dopamine on both Helix inhibitory and excitatory receptors (Walker et al., 1980). At one time apomorphine was considered the 'classical' dopamine agonist but it has now been found that it acts only as a partial agonist and even as an antagonist at certain vertebrate and invertebrate dopamine receptors (Kebabian and Calne, 1979; Walker et al., 1980).

Some neuroleptic drugs provide the most potent dopamine antagonists. These include butyrophenones (for example, haloperidol); thioxanthines (for example, cis-thiothixene); and substituted benzamides (for example, sulpiride). The potency of these inhibitors depends on the dopamine receptor involved. Butyrophenones have nanomolar potency at vertebrate central D-2 receptors; micromolar potency at central D-1 receptors; in the periphery they were found to be of limited use (Leff and Creese, 1983; Golberg and Kohli, 1979). Thioxanthines are more potent inhibitors of vertebrate central D-1 than D-2 receptors (Leff and Creese, 1983). For vertebrate peripheral receptors sulpiride is a major antagonist (Golberg and Kohli, 1979). In Helix fluphenazine, (+)-butaclamol, cis-flupenthixol and sulpiride have all been found to block both the excitatory and the inhibitory responses of dopamine (Walker et al., 1980). Ergometrine has been shown to be a potent blocker of dopamine inhibition in Helix (Walker et al., 1980), but acts as a partial agonist at the cockroach salivary gland (House



and Smith, 1980). LSD (D-lysergic acid diethylamide) is also a potent dopamine inhibitory receptor blocker in *Helix* (Woodruff, Walker and Kerkut, 1971), but Nathanson and Greengard (1974) found it to be ineffective in inhibiting dopamine-stimulated adenylate cyclase in cockroach thoracic ganglia homogenates.

Evans (1981b) carried out a detailed agonist/antagonist study of the octopamine receptors at the locust neuromuscular junction. By comparing the actions of several drugs he concluded that there are three different pharmacological classes of octopamine receptor. Effective agonists included clonidine, tolazoline, tramazolin and naphazoline. The most potent antagonists included phentolamine, promethazine, chlorpromazine, yohimbine and metoclopramide.

#### Possible Involvement of Cyclic AMP

Since the work on cockroach salivary gland (Grewe and Keabian, 1982; Gray, Ginsborg and House, 1984), central nervous system homogenates (Nathanson and Greengard, 1974; Harmar and Horn, 1977) and also on locust neuromuscular junctions (Evans 1984a and b) have provided evidence that both dopamine and octopamine act through cyclic AMP, it was thought that the dopamine response at D3 might also be mediated in this way.

IBMX is a phosphodiesterase inhibitor and hence its addition would be expected to potentiate any cyclic AMP dependent process. Gray, Ginsborg and House (1984) found that IBMX

alone produced a dose dependent fluid secretion at the cockroach salivary gland. Reservations about the site of action of IBMX in this preparation have already been stated (see Introduction). Positive evidence for the utility of IBMX in the investigation of cyclic AMP dependent processes in insects has been provided by Evans (1984a and b). It had already been shown that the extensor tibiae muscle of the locust hindleg has a single octopamine-containing neurone as part of its innervation (Evans and O'Shea, 1977 and 1978; O'Shea and Evans, 1979; Hoyle, 1975). Evans (1984a and b) showed that: (i) the action of octopamine is coupled to cyclic AMP production; (ii) IBMX alone (10  $\mu$ M) had octopamine-like effects; and (iii) in the presence of lower concentrations of IBMX (1  $\mu$ M) the octopamine-mediated response was potentiated. Even though it was ensured that the dopamine response at D3 was not maximal it was not potentiated by IBMX and in some cases was actually decreased. It is known that IBMX is an antagonist at adenosine receptors (Wolff, Londes and Cooper, 1981) and it is possible that dopamine could be acting at these, although evidence provided below will show that this is unlikely.

Dibutyryl cyclic AMP is a membrane soluble cyclic AMP analogue and so would be expected to mimic cyclic AMP dependent processes. Gray, Ginsborg and House (1984) found that cyclic AMP itself caused a dose dependent secretory response of cockroach salivary glands; Berridge (1972) found the same with blowfly salivary glands. However, Evans (1984a) found that neither cyclic AMP nor dibutyryl cyclic

AMP (up to 10 mM) produced any consistent effects on the locust extensor preparation, although another derivative, CPT cyclic AMP (8-(4-chlorophenylthio)adenosine 3':5' monophosphate), did mimic octopamine. Further increases were observed when IBMX was also included. In the D3 preparation dibutyryl cyclic AMP did not mimic the dopamine response. The two main problems with cyclic AMP and its analogues is their restricted permeability and their rapid metabolism by phosphodiesterase. The inclusion of IBMX in the bathing medium of D3 excludes the latter problem. It is possible though that dibutyryl cyclic AMP has limited membrane permeability in this preparation, which could be checked by recording the effect of CPT cyclic AMP, or by eliminating the permeability problem altogether by intracellular injection of cyclic AMP. One important point, however, is that cyclic AMP and its analogues can act at adenosine receptors (Wolf, Londes and Cooper, 1981). If dopamine was acting at adenosine receptors then dibutyryl cyclic AMP (1 mM was used) would be expected to mimic the response of bath applied dopamine. At a 10 fold lower concentration than dibutyryl cyclic AMP dopamine rapidly caused a maximum depolarization in D3 (further addition of dopamine to the surface of D3 produced no effect). Dibutyryl cyclic AMP gave no depolarization. Hence it is unlikely that dopamine is acting through adenosine receptors on D3.

The diterpine forskolin is believed to stimulate cyclic AMP production by directly activating the catalytic subunit of adenylate cyclase (Seamon and Daly, 1983). Evans (1984a and b) found that forskolin (15  $\mu$ M) increased cyclic AMP levels

in extensor muscle and increased the same three parameters as octopamine during the firing of the slow motoneurone SETi. In this study on D3, 20 and 50  $\mu$ M forskolin did not depolarize the cell or affect the dopamine response. As with IBMX and dibutyryl cyclic AMP it was always ensured that the dopamine response was not maximal. Forskolin did increase the firing frequency of the action potentials recorded from D3 (Fig. 2.26a). This could be for either of the following reasons:

1. A direct action on the excitability of a region of D3 other than the cell body (for example, the spike initiation zone).
2. A presynaptic action potentiating synaptic activity onto D3.

At present there is no evidence available to determine which mechanism is operating, although any synaptic effects were clearly not profound enough to be recorded as postsynaptic potentials in the cell body.

Although the results suggest that at D3 the dopamine response is not mediated through cyclic AMP, nothing can be said about the way in which dopamine acts at other neurones in the cockroach CNS. Since biochemical experiments have indicated that a dopamine-sensitive adenylate cyclase is present in the insect CNS, it is likely that, as in vertebrates and other invertebrates, some dopamine receptors are linked to adenylate cyclase, while others are not.

SUMMARY

In summary the results of the present study suggest that:

(1) The cell body of the common inhibitory motoneurone, D3, possesses receptors that respond to catecholamines and octopamine.

(2) Activation of these receptors causes a relatively long lasting depolarization (40-60 sec).

(3) The response to dopamine does not appear to be mediated through activation of adenylate cyclase.

APPENDIX

THE NEUROANATOMY OF THE SUBOESOPHAGEAL GANGLION.

INTRODUCTION

In 1974 Gregory described the basic framework of the cockroach mesothoracic ganglion and characterized most of its peripheral roots. A few years later, Tyrer and Gregory (1982) described the organization of the thoracic and suboesophageal (SOG) ganglia in the locust. They found that each ganglion in the locust was constructed to a common plan which was similar to that found by Gregory (1974).

During the course of this study several types of experiment were carried out using the SOG and it was therefore decided that knowledge of its basic neuroanatomy would aid interpretation of the results. It has already been noted (Chapter I, section III) that the basic framework of this ganglion is quite different from the three thoracic ganglia and it was uncertain how this would affect the internal structure. It would also be useful for the interpretation of sectioned ganglia since once a neurone has been functionally identified the anatomical position of the branching could be related to other anatomical features. This would allow possible relationships to be established between stained neurones in different preparations (see Tyrer and Gregory, 1982).

MATERIALS AND METHODS

SOG ganglia of adult male cockroaches were dissected out as described before (Chapter I, section I). They were fixed overnight in 4% formol saline and then dehydrated, cleared, infiltrated and embedded in Paraplast (BDH Chemicals) as follows:

- (1) 1 hr in 50% ethanol.
- (2) 0.5 hr in each of 70%, 80%, 90%, 95%, 100%, 100% ethanol.
- (3) 0.5 hr in toluene.
- (4) 0.75 hr in each of 3 changes of Paraplast and then embedded.

Next sections were cut at 10  $\mu$ m in the three most usual planes, transverse, horizontal and sagittal.

The staining solution was made up as follows (see Hill, 1964):

- (1) A stock solution containing 0.05% Basic Fuschin in 50% acetone/ 50% distilled water was prepared and used when required.
- (2) Immediately before use 0.1 g solid borax was added to 100 ml of the stock solution, stirred for about 10 min and then filtered.

The sections were stained as follows:

- (1) Xylene, 2 x 5 min.
- (2) 100 % ethanol, 2 x 2 min.
- (3) 2 min in each of 95%, 70% and 30% ethanol.
- (4) Distilled water, 3 min.
- (5) Basic Fuschin staining solution, 2-4 min.



- (6) 10 mM sodium acetate pH 4.0 (destaining solution), 30 sec - 1 min, keep dipping and observing.
- (7) Distilled water, 3 min.
- (8) 2 min in each of 30%, 70% and 95% ethanol.
- (9) 100% ethanol, 2 x 2 min.
- (10) Xylene, 2 x 3 min.

The slides were then mounted in DPX.

The sections were observed using the Leitz Dialux 20 set-up and photographed onto Ilford FP4 film (see Table 1.4 for developing procedure). Drawings were made from the negatives by first projecting them to the desired size using an enlarger.

## RESULTS

### Organization of the Ganglion Core

The general structure of a cockroach ganglion has previously been described in the General Introduction. The central core is mainly composed of fibre tracts and neuropile sensu stricto. The tracts include sensory fibres from peripheral nerves, fibre bundles from cell body groups and through fibres from other ganglia. The neuropile sensu stricto is the finer fibrous area which lies between the tracts. It is here that terminal arborizations and synapses are found. On studying cockroach thoracic ganglia Pipa, Cook and Richards (1959) found a region of especially fine neuropile ventrally in each ganglion core. This they termed the ventral association centre (VAC). The most useful landmarks for examining the core are the longitudinal tracts, which run between the interganglionic connectives, and the commissures (transverse tracts) which link the two halves of the ganglion together. The terminology used to describe the tracts here is essentially the same as that employed by Pipa et al. (1959), Gregory (1974) and Tyrer and Gregory (1982).

### Suboesophageal Ganglion (SOG)

This ganglion consists of three fused neuromeres. From anterior to posterior these are: the mandibular (Md), the maxillary (Mx) and the labial (Lb) neuromeres. The ganglion innervates the mouthparts, neck muscles, salivary glands and receives fibres from some sensory areas of the head and neck. There are nine pairs of peripheral nerves and in this study they have been numbered in the same order as that used

by Guthrie and Tindall (1968). This differs from the order used for the locust SOG (Tyrer and Gregory, 1982; Altman and Kien, 1979). Each neuromere contains one large nerve which innervates the mouthparts (Fig. A1): mandibular (MdN or N3); maxillary (MxN or N4), this has a ventral and a smaller dorsal root; and labial (LbN or N5). N1 (hypopharangeal nerve) is a fine nerve which originates near the midline. N2 (not shown) arises slightly dorsal to N1 and runs to the corpus allatum. N6 and N7 are small nerves which are thought to innervate cervical muscles. N8 lies more posterior to these and runs to the salivary gland. N9 is another fine nerve which runs posteriorly to the prothoracic glands.

Sections of the SOG are shown in Figs. A1, A2 and A3. A list of abbreviations used is located at the end of this section.

#### Transverse Sections

Although the SOG is made up of three neuromeres it is smaller than the first two thoracic ganglia which contain only one. It is only the labial neuromere which in any way resembles the 'unit' neuromere of the other two ganglia (cf Fig. A1.A-E with Fig. 2 Gregory, 1974). The other two are considerably reduced and compressed and this makes it fairly difficult to delimit them. On careful examination of the sections it was decided that the mandibular neuromere is located above the labral neurones in the centre of the anterior part of the ganglion (see Fig. A1.I-L and Fig. A3, E and F). The maxillary neuromere starts from the anterior end of the ganglion, on either side of the mandibular one, and extends, dorsally, to about half way through the

circumoesophageal connectives (COC); ventrally, a commissure is the end point (Fig. A1.G-L). The labral neuromere extends ventrally and dorsally from the latter two points to the posterior end of the ganglion (Fig. A1.A-E and Fig. A3).

Fig. A1.A-E shows that the labial neuromere contains longitudinal tracts equivalent to those found in the other thoracic ganglia, plus a few additional ones which presumably link this ganglion to the brain (cf. Fig. A1 and Fig. 2 Gregory, 1974). This situation was also found by Tyrer and Gregory (1982) in the locust. After Fig. A1.E the fibres turn upwards into the COC and from this point it becomes very difficult to identify them. The labial neuromere contains six dorsal (DCI - DCVI) and three ventral (LbVCI - LbVCIII) commissures and two areas of dense neuropile (pVAC and aVAC). Fig. A1.G shows substomodeal commissure 1 which contains most of the tritocerebral fibres that cross the midline. This confirms Willey's (1961) observation that the commissure and ganglion are separate. Fig. A1.G also shows, however, that there are commissural fibres which run between the COCs, their origin is not known. The labral cell bodies, along with their tracts and commissure are seen in Fig. A1.I. Since the maxillary and mandibular lie mainly behind the COCs they do not have the longitudinal tracts running through them, although they both send fibres into, (and presumably receive fibres from), these connectives. These two neuromeres are so reduced in size that it was impossible to recognize commissures that are analogous to those seen in the labral neuromere. Hence, those that are described are labelled without numbers.

### Horizontal Sections

The horizontal sections are the hardest to interpret and are the least useful in providing information about ganglionic structure. Unlike the SOG of the locust (see fig. 12 Tyrer and Gregory, 1982) it is not possible to totally expose longitudinal tracts due to the turning of these into the COCs; they could only be identified in the posterior half of the labial neuromere (Fig. A2.C-H). Fig. A2.D-H clearly shows the labral neurones are present right through the ganglion from the ventral to the dorsal surface. The positions of most of the commissures in relation to the roots of the three main nerves (LbN, MxN and MdN) can also be seen (Fig. A2.A-H).

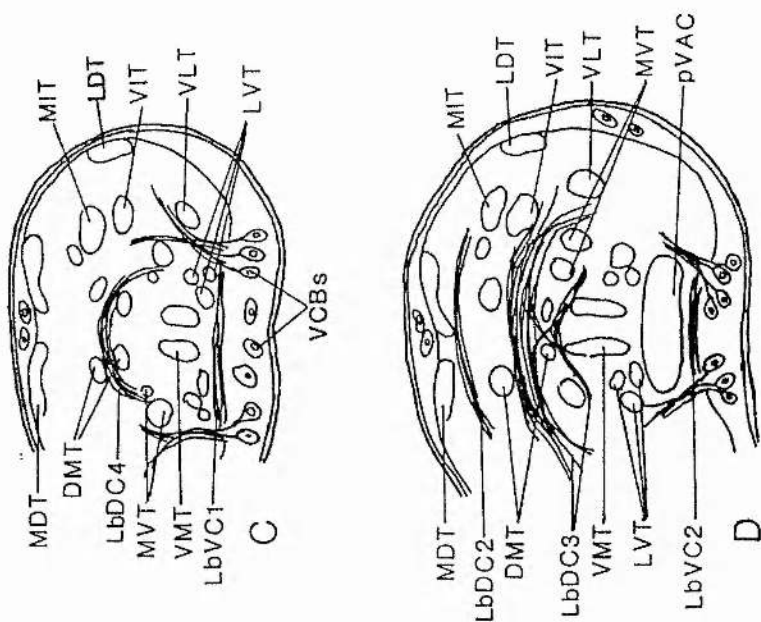
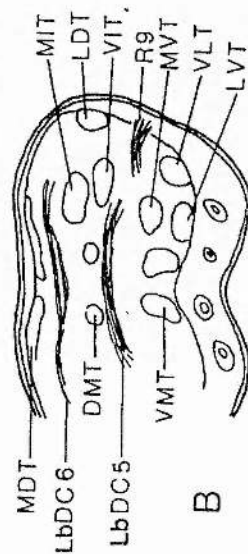
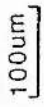
### Sagittal Sections

These sections are easiest to interpret near to the midline (Fig. A3.F). The labral cell bodies are clearly seen, as are the commissures and the DUM neurones. The central part of the mandibular neuromere is situated anterior to the labral neurones. Although the maxillary and labial neuromeres are less easy to distinguish, especially at the dorsal surface, with the aid of the diagrams of the transverse sections (Fig. 1A.F,G), the two areas can be separated. As the sections progress from the edge of the ganglion towards the centre, first the labial, then the maxillary and finally the mandibular roots are observed (Fig. A3.A-E). Several of the longitudinal tracts were also distinguishable (Fig. A3.C-E).

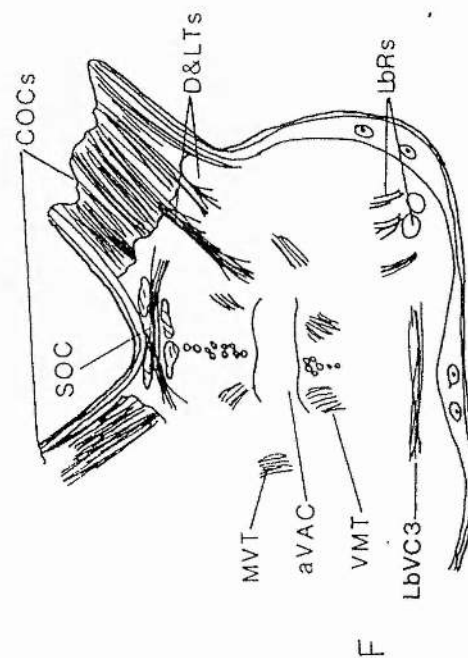
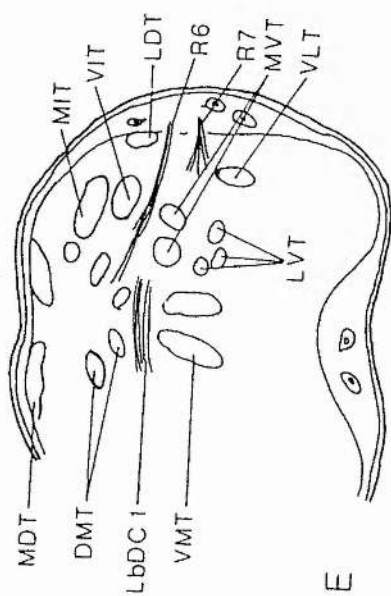
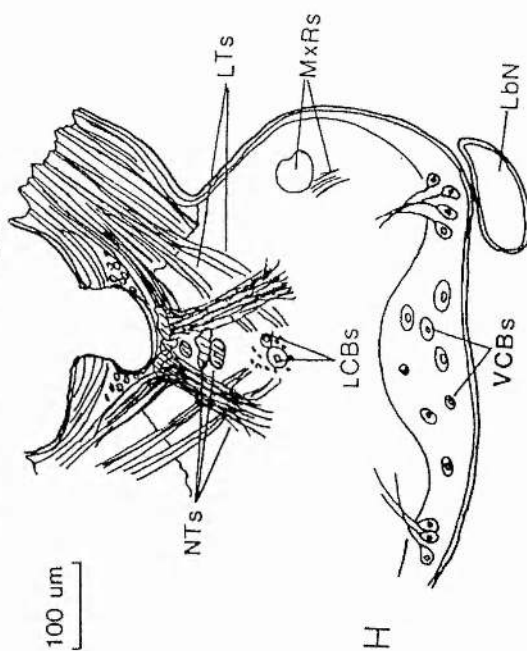
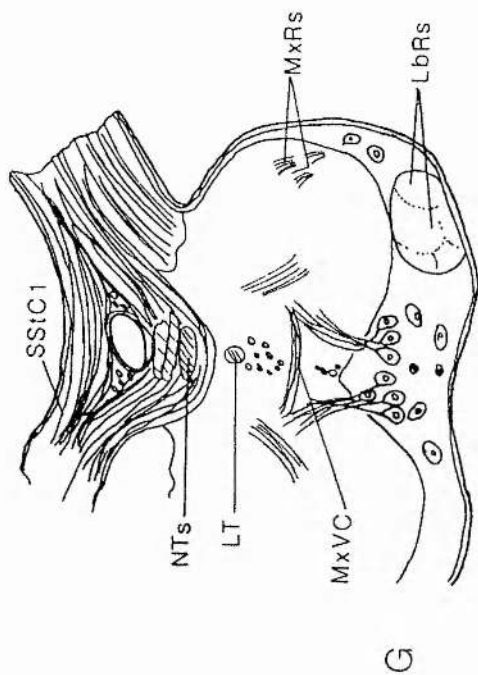
## LIST OF ABBREVIATIONS

A	anterior
aVAC	anterior ventral association centre
CF	commissural fibre
COC	circumoesophageal connective
D	dorsal
DIT	dorsal intermediate tract
DMT	dorsal medial tract
DT	dorsal tract
DUM	dorsal unpaired neurone
L	lateral
LbDC1-6	labial dorsal commissure 1-6
LbN	labial nerve
LbR	root of labial nerve
LbVC1-3	labial ventral commissure 1-3
LC	labral commissure
LCB	labral neurone
LDT	lateral dorsal tract
LT	labral tract
LVT	lateral ventral tract
M	medial
MdC	mandibular commissure
MdN	mandibular nerve
MdR	root of mandibular nerve
MDT	medial dorsal tract
MVT	medial ventral tract
MxC	maxillary commissure
MxN	maxillary nerve
MxR	root of maxillary nerve
N1/6-9	nerve 1/6-9
N1R	root of nerve 1
NT	nerve tracts
P	posterior
PC	posterior interganglionic connective
pVAC	posterior ventral association centre
R6-9	root of nerve 6-9
SOC	suboesophageal commissure
SStC1	substomadeal commissure 1
V	ventral
VCB	ventral cell body
VIT	ventral intermediate tract
VLT	ventral lateral tract
VMT	ventral medial tract

Figure A1.A-L Transverse sections of a cockroach suboesophageal ganglion showing the chief neuroanatomical features. The section planes (A-L) are shown in a lateral view of the ganglion shown at reduced scale at the top left.







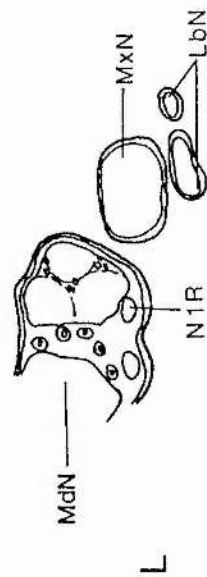
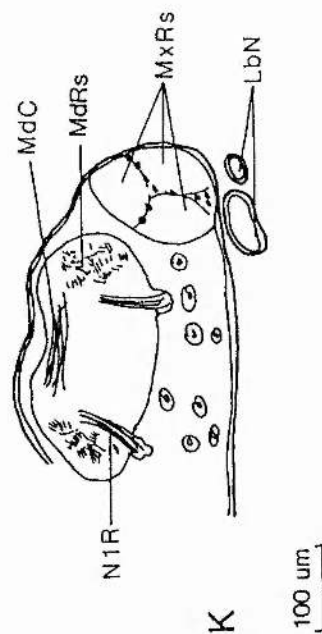
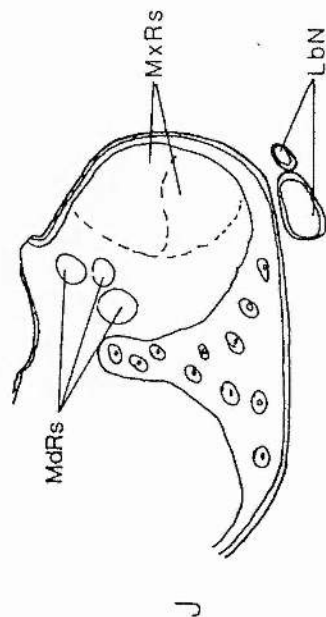
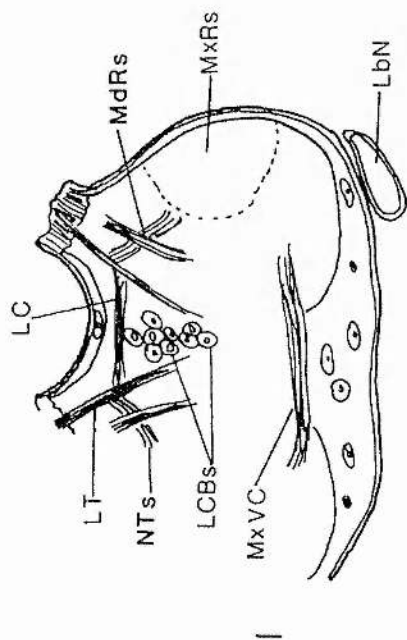
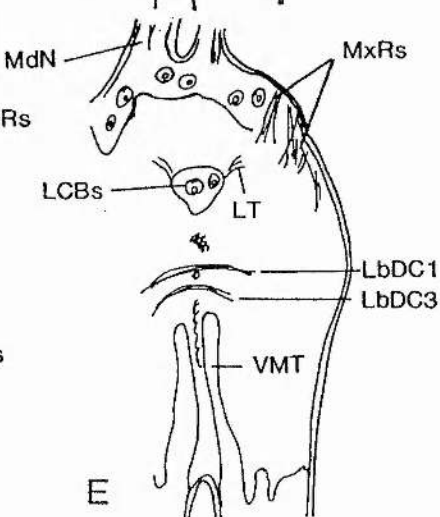
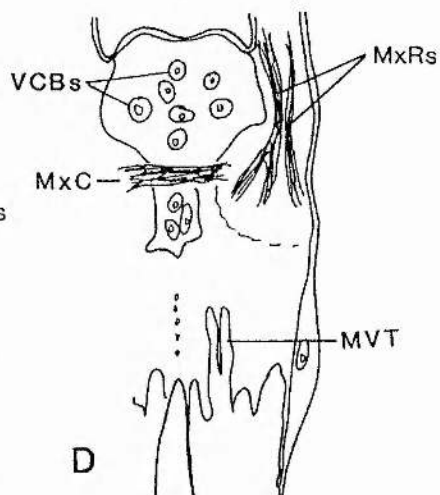
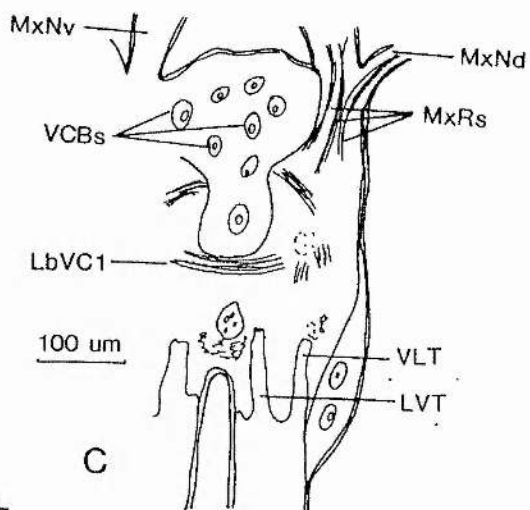
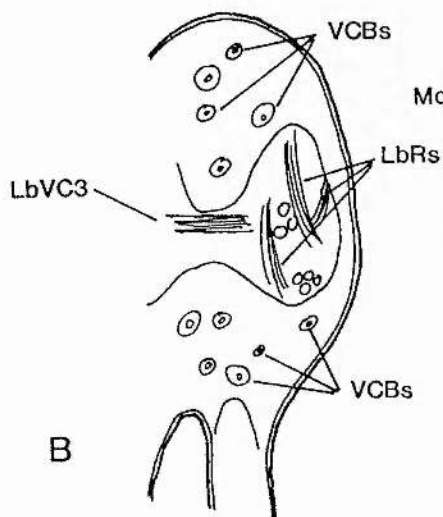
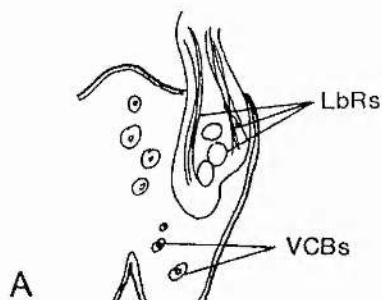
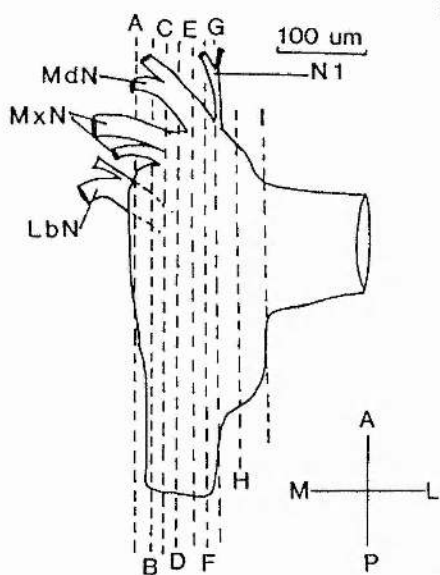


Figure A2.A-I Horizontal sections of a cockroach suboesophageal ganglion showing the main neuroanatomical features. The section planes (A-I) are shown in a lateral view of the ganglion shown at reduced scale at the top left.



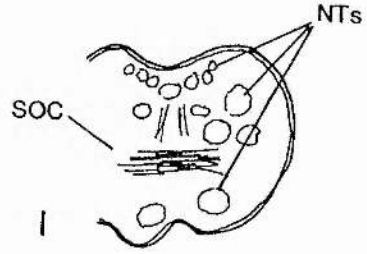
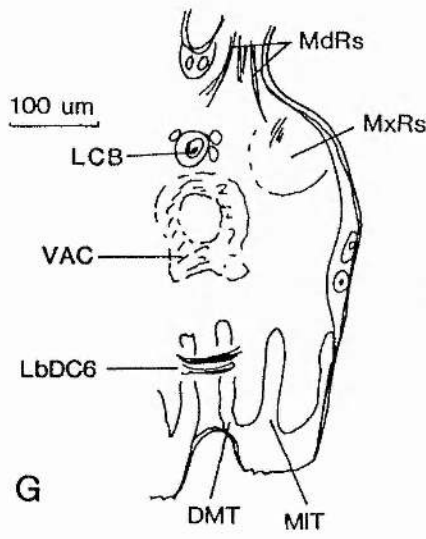
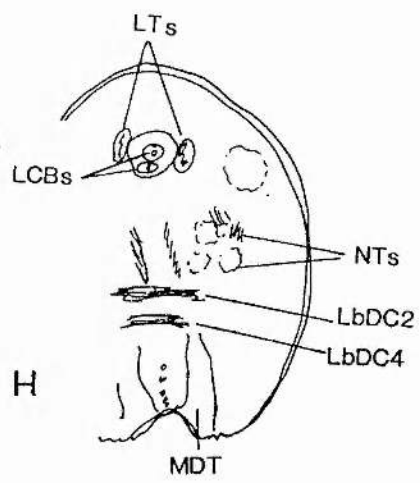
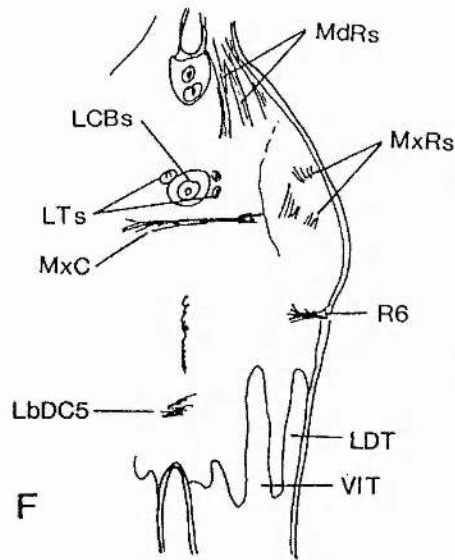
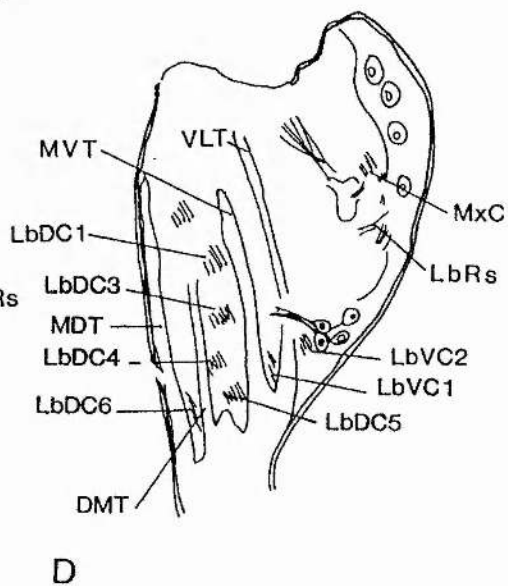
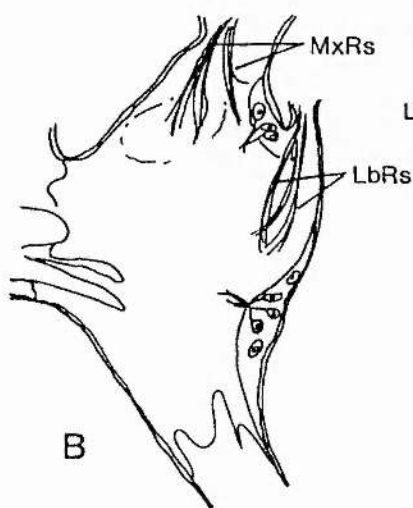
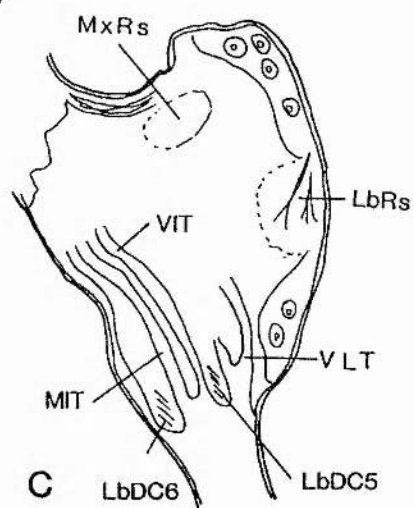
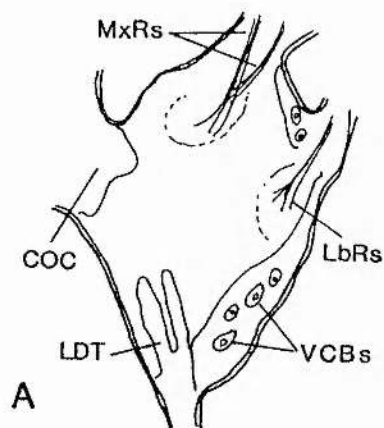
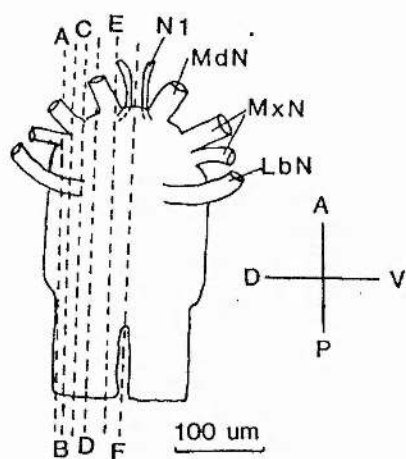
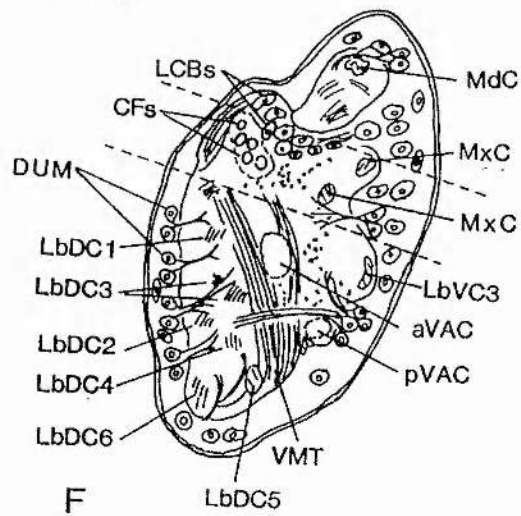
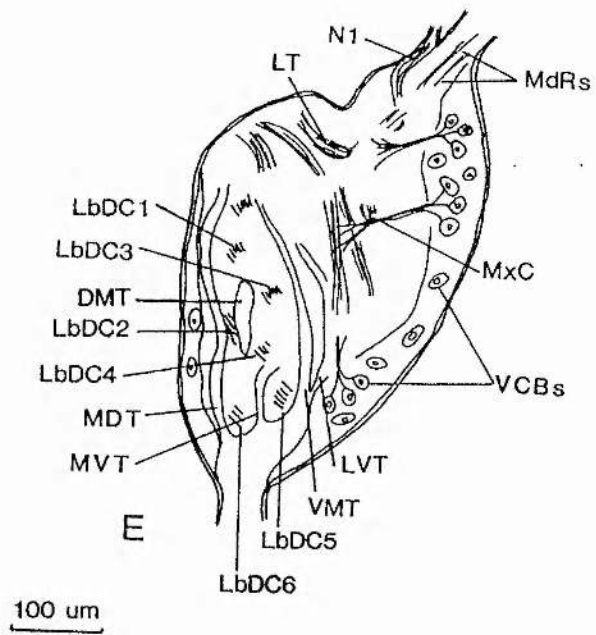


Figure A3.A-F Sagittal sections of a cockroach suboesophageal ganglion showing the main neuroanatomical features. The section planes (A-F) are shown in a ventral view of the ganglion shown at reduced scale at the top left.







DISCUSSION

It has been shown that out of the three neuromeres that make up the SOG only the labial one approaches the complexity of the mesothoracic 'unit' neuromere. It is only after sectioning that the boundaries of the neuromeres can be identified.

The dorsal unpaired catecholamine-containing neurone (Chapter I, section I) is one of the labral cell bodies (Fig. A3.F). Evidence indicated that it sends its axon into the COCs, the observation of a pair of labral tracts (Fig. A1.I) supports this.

Varicose fibres from the pair of salivary duct nerves (Chapter I, section II) were concentrated on the dorsal surface between the COCs. Fig. A1.F-H shows that there are many fibres (including some from the mandibular neurones and descending cerebral neurones) densely packed in this area and it is likely that these functionally different neurones interact at this point.

One of the main drawbacks of using sectioned tissue to determine the location of neurones and fibres containing certain putative transmitters is that it can be difficult to relate the results to the ganglion as a whole. If, however, anatomical information is available such that tissue landmarks can be identified, it will be much easier to see the results at the whole ganglion level and to collate the results of different putative transmitters.

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